BEHAVIORAL CHANGE IN RESPONSE TO LOW-GRADE NON-INFECTIOUS STIMULI: FINDINGS AND POTENTIAL NEUROIMMUNE MECHANISMS

BY

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DISSERTATION

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ABSTRACT

Individuals afflicted with a pathogenic infection exhibit symptoms including lethargy, malaise, listlessness, loss of interest in social and environmental surroundings, and anorexia. Together, these symptoms comprise what is recognized as "sickness behavior." Sickness behavior represents a conserved, motivational behavioral state that, with the induction of fever, serves to help an organism combat infection and ultimately survive. Long dismissed as an unavoidable consequence of the physiological changes resulting from pathogen-dependent immune activation, it is now well known that sickness behavior is a consequence of neuroimmune activation. Neuroimmunity serves as a bridge between the peripheral immune system and the central nervous system, acting to signal bi-directionally both centrally and humorally between these two systems. Proinflammatory cytokines, most often originating from innate immune cells, are the principle signaling molecules from the periphery to the brain. Sickness behavior is typically transient, resolving upon pathogen clearance. Exacerbated or unchecked proinflammation, such as occurs in chronic disease or autoimmune disorders, leads to maladaptive behaviors such as depression and anxiety. Neuroimmune activation often also causes cognitive impairments in addition to sickness, depressive or anxietal behaviors. It is widely recognized that non-infectious stimuli, such as ionizing radiation or hypoxia can activate the neuroimmune system and cause sickness behavior and other behaviors. This literature review focuses on the different ways activation of the neuroimmune system can occur, the inflammatory and behavioral consequences of its activation and some of the modulators of neuroimmune communication. Chapters 2 and 3 will provide evidence showing low dose ionizing radiation and chronic low-grade hypoxia can result in the display of typical neuroimmune-mediated behaviors, as well as other physiological changes. Taken together, these findings show that behavioral change can be induced by lower doses of ionizing radiation and hypoxia than have previously

been reported, and that neuroimmune signaling can occur where and when it was previously unknown to.

To my mother, father and stepfather.
Throughout my life you have always shown me what it means to work hard and put your best effort forward. I will carry these lessons and your words of wisdom with me for the rest of my life. Thank you for your unending love, support and encouragement.

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CHAPTER ONE[a]

LITERATURE REVIEW

I INFLAMMATION AND NEUROIMMUNE ACTIVATION

Neuroimmunology as a field of study is relatively new with respect to the individual fields of neuroscience and immunology. However, that bi-directional communication can occur between the immune and nervous systems was known decades before the introduction of neuroimmunology as a term and field of study (1). Neuroimmunity is an important component of the innate immune system that acts to communicate peripheral immune status to the brain (2-3). In response to host infection, accessory immune cells (e.g. macrophages, dendritic cells) produce soluble mediators capable of signaling other immune cells, which then become activated and involved in fighting infection, often producing additional signaling molecules. The soluble mediators elicited by host infection are proinflammatory cytokines – small peptides involved in both intra- and intercellular communication, relay of information regarding development, tissue repair, hematopoiesis, inflammation and innate and adaptive immune responses (2,4). The brain has long been considered an immunologically privileged organ – that is to say foreign antigens within the brain parenchyma can avoid systemic immunological recognition – but the definition continues to be challenged as new findings arise. It is well recognized that the brain's immune status can vary largely depending on brain region as well as natural processes such as aging (2,5). The brain contains its own immune cells, such as dendritic cells and specialized brain

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macrophages known as microglia. These brain-based immune cells respond to inflammatory stimuli by producing proinflammatory cytokines that can act on other immune cells and both neuronal and non-neuronal cells in the brain (2). While these actions are similar to those of the peripheral immune response, they differ in that molecules produced by activated brain immune cells do not lead to recruitment and invasion of peripheral immune cells into the parenchyma (2,5). Proinflammatory cytokines act on brain via several different routes. Initially it was discovered that peripheral proinflammatory cytokines, especially IL-1β, bind to afferent nerve fibers (e.g. vagus nerve), leading to increased vagal signaling to the brain. Cytokines may act on the brain by volume diffusion at sites outside the blood brain barrier (BBB), like the circumventricular organs (CVOs). Cytokines also exert their effect on the brain through saturable transport systems at the BBB, and perivascular macrophages and endothelial cells in brain vasculature possess receptors for IL-1β, and its binding causes production of prostaglandin E2, which ultimately induces the febrile response (2,6-7) Cytokines produced by peripheral innate immune cells signal their status to the central nervous system (CNS), which elicits a neuroendocrine response via the hypothalamic-pituitary-adrenal (HPA) axis (4,8). The activated HPA axis signals both peripherally to help regulate the immune response to infection, as well as centrally to elicit additional physiological effects (6-7,9). Major proinflammatory cytokines include interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (2-4,6-7). These cytokines have pleiotropic activity and functional redundancy. Proinflammatory cytokines can interact with a variety of cell types, including neurons, and induce a response specific to a cell type (2,4). Cytokines can also influence the production and response(s) to other cytokines – a result of an intertwining complex network of signaling pathways exerting different influences on each other (4). Proinflammatory cytokines rarely act alone. For example, IL-1β

secretion is often accompanied by the release of other proinflammatory cytokines (e.g. IL-6 and TNF-α), emphasizing their redundant function (10). Upon activation of the HPA axis, adrenocorticotropic hormone (ACTH) is released from the anterior pituitary gland into the circulation where it binds cells of the adrenal cortex leading to the release of glucocorticoids (GCs) (7,9). GCs have multiple physiological functions, but critical to neuroimmunity they can inhibit proinflammatory cytokine production and inflammation, and can also act via negative feedback on the HPA axis to halt its activation (9,11-12). GCs also act on immune cells to increase the synthesis of anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1RA) and interleukin-1 receptor II (IL-1RII) (11,13). Together, the suppression/inhibition of proinflammatory cytokines and induction of anti-inflammatory cytokines by GCs leads to the eventual cessation of the inflammatory response and return to homeostasis in healthy individuals.

II NEUROIMMUNE ACTIVATION-INDUCED BEHAVIORAL CHANGE

A) SICKNESS BEHAVIOR

Proinflammatory cytokine release in response to peripheral infection can induce behavioral change via a neuroimmune mechanism. Individuals afflicted with bacterial or viral infection, or treated with lipopolysaccharide (LPS; a component of gram negative bacterial cell walls and a potent activator of the neuroimmune system) show symptoms of lethargy, malaise, listlessness, loss of interest in social activity, and reduced food and water consumption. Together, these symptoms comprise what is referred to as “sickness behavior” (2-3,6,14). Sickness behaviors for many years were ignored by physicians as an unavoidable consequence of

the physiological changes caused by pathogen-dependent immune activation. Hart was among the first to propose behavioral change is not a consequence of the general debilitative effects of infection or a maladaptive response, but rather a motivational state with the function of potentiating the febrile response to infection, thus facilitating the recovery and survival of the host. This is supported by the fact that, in addition to fever, the behaviors associated with an infection are ubiquitous among most animal species (3,6-7). Hart's seminal work in sickness behavior has led to an abundance of additional research toward understanding its role in host recovery/survival and the underlying mechanisms mediating behavioral change.

Proinflammatory cytokines are principle inducers of sickness behavior, both those endogenously produced in response to immune stimulation, as well as those exogenously administered (peripherally or centrally) to experimental animals (2-3,6-7,14-16). Peripheral proinflammatory cytokine signaling to the brain induces central production of proinflammatory cytokines, and this can occur via the neural, humoral, or membrane transporter routes (2,6-7,14-16). Immune cells in the brain respond to much lower concentrations (100-1000 times lower) of cytokines than do those in the periphery, additional evidence that sickness behavior is a consequence of brain-based immune activation (14). The initial discovery and subsequent investigation of sickness behavior and its neuroimmune-mediated mechanism(s) has led to the discovery and expansion of study to behaviors occurring after sickness behaviors have otherwise resolved.

B) DEPRESSIVE-LIKE BEHAVIOR

In individuals with chronic illnesses such as autoimmune disorders and cancer, the peripheral immune proinflammatory response can continue unabated. Interestingly, major depressive disorders are more commonly observed in patients with chronic inflammatory

diseases than in those without disease. Depression differs from sickness behavior in that it does not serve to assist the host in combating and recovering from an infection, but is considered a maladaptive consequence of unchecked proinflammatory signaling (2,17). In animals, "depressive-like" behaviors are studied. These behaviors mimic some of the features of clinical depression such as helplessness and anhedonia (2). Depressive-like behavior has been observed in rodent models at least as far back as the mid 1990s, when Yirmiya reported depressive-like behavior in rats following LPS administration (18). Importantly, depressive-like behavior is generally observed following recovery from the more acute sickness behavior. This is known to occur following initial sickness behavior responses to LPS (acute immune activator), and *Bacillus Calmette-Guerin* (chronic immune activator) (17,19-21). As with sickness behavior, proinflammatory cytokines can also induce depression, findings initially observed in human longitudinal studies where proinflammatory cytokines were administered as immunotherapeutics. In these studies all patients experienced sickness behavior following the first cytokine treatment, but after its resolution, up to one third of patients develop major depressive disorders (2,21). Further evidence for proinflammatory cytokine-induced depressive-like behavior comes from rodent studies in which depressive-like behavior was induced by IL-1β treatment (2,22). Important to all studies investigating the impact of proinflammation on the development of depressive- or depressive-like behavior is to validate the behavior as depressive by pre-treating with antidepressant agents. In both animals and humans pretreatment with antidepressants before the proinflammatory cytokine treatment leads to reduced severity of depressed mood (23).

C) COGNITIVE BEHAVIOR

The proinflammatory response can also lead to cognitive impairment in the form of hippocampal-dependent memory deficits (2,10,24-27). Tests like contextual fear conditioning, related to hippocampal long-term potentiation, are affected by increased proinflammatory cytokine levels in the periphery and brain (2,10). Non-hippocampal-dependent memory functions remain unaffected following neuroimmune activation and proinflammatory cytokine release. It appears that IL-1β is the likely critical proinflammatory cytokine involved in memory deficits, as the dentate gyrus of the hippocampus possesses IL-1 receptors (24). This doesn't rule out the involvement of other proinflammatory cytokines, as Pugh et al. indicated in their review on IL-1β and memory function, TNF-α may have similar effects on memory impairment (10). The detrimental effects of proinflammation on cognition appear to be largely in the consolidation of memories, but not the learning process itself. An example of this comes from studies using the Morris water maze (MWM), in which intracerebroventricular (ICV) IL-1β treated rats were able to learn the escape task, but were impaired in their ability to recall the location of the escape platform (26). Recently, Goshen et al. published findings showing that IL-1β is actually required for learning and memory formation, and only when its levels are perturbed beyond a normal physiological range do hippocampal memory deficits occur (27-28). Il-1β's effects on memory consolidation, both normal and impaired, can be reversed in either direction via treatment with IL-1RA (10,27-28).

D) ANXIETY-LIKE BEHAVIOR

Similar to depression and depressive-like behavior, anxiety and anxiety-like behavior can be induced by proinflammatory cytokines in humans and animals, respectively (23).

Proinflammatory cytokine-induced development of anxiety-like behavior is less studied than sickness, depressive-like and cognitive behaviors. In one study aimed at determining whether anxiety-like behavior is a direct result of increased brain-based proinflammatory cytokine levels, Swiergiel and Dunn reported that LPS or IL-1β led to decreased open arm entries in an elevated plus maze (EPM) and decreased line crossings within the central area of an open field test (OFT) arena, both indicative of anxiety-like behavior. These decreases were also accompanied by significant reductions in arm entries in the EPM and total number of line crosses in the OFT, indicating sickness behavior may have clouded the effects of LPS and IL-1β on anxiety-like behavior (29). Anxiety-like behavior in rodents is largely studied in response to acute or chronic stress paradigms, which still involves the neuroimmune system in several ways.

III STRESS AND NEUROIMMUNITY

A) BASIC MECHANISM OF THE STRESS RESPONSE

In addition to neuroimmune signaling, the physiological responses to acute and chronic psychological stress also utilize the bi-directional communication pathways between the CNS and the immune system, and can likewise impose alterations in neuroimmune signaling and response to infection. There are many definitions of psychological stress, but to define it in its simplest terms relevant to neuroimmunology, psychological stress occurs when events or environmental demands exceed an individual's perceived ability to cope. Depending on the duration and course, psychological stress can affect the immune system in different ways (30). When an event is perceived as stressful, the HPA axis is activated via CRH release from the paraventricular nucleus (PVN) of the hypothalamus. An additional relevant system activated by

psychological stress is the sympathetic-adrenal-medullary (SAM) axis (30-32). Together these systems react to stress by releasing pituitary and adrenal hormones, which include ACTH, GCs, catecholamines (adrenaline and noradrenaline) and growth hormone, among others (30). These hormones have potent and often times multifunctional effects on the body's physiology and the immune system is not excluded from the list of targets these hormones can act on (30-31).

B) ACUTE STRESS

There is evidence for both immunoenhancing, as well as immunosuppressive effects in response to psychological stress (33). Most immune cells express receptors for one or more of the hormones associated with the HPA and SAM axes, and the effects of receptor binding can act either directly, via binding to its analogous receptor, or indirectly through disruption of cytokine production (30). Immunoenhancing effects of stress are largely restricted to acute stress, where acute activation of the HPA axis and the sympathetic nervous system (SNS) during the "fight or flight response," leads to increased leukocyte trafficking and enhancement of B cell immune responses. This is postulated to occur as a protective mechanism against wound infection following an aggressive encounter (32). Most acute psychological stress is associated with immunosuppression, or a shift to an anti-inflammatory phenotype. For example, in a study involving medical students taking an exam, acute stress led to a shift in CD4+ T helper cells (Th) from a Th1 (proinflammatory) to a Th2 (anti-inflammatory) phenotype. This may result in dysregulation of immune responses (34). Responsiveness to vaccines is also reduced by increasing levels of psychological stress, which is interpreted as a proxy for responses to infectious agents (31). Further evidence for psychological stress' suppression of immune activity from animal studies has shown that stress causes reductions in vaccine response, enhances viral

and bacterial pathogenesis, alters autoimmune disease course and increases wound healing time. These effects are associated with decreased proinflammatory cytokine production and reduced immune cell trafficking (30-31).

C) CHRONIC STRESS

Chronic stress, on the other hand, can lead to increased proinflammation. In a model of social disruption, in which a dominant intruder was repeatedly introduced to a cage of mice (i.e. chronic psychological stress), submissive mice developed GC resistance in peripheral immune cells, rendering them insensitive to the effects of GCs (35). In general, chronically stressed individuals, as is seen in depression, may develop GC resistance and mount exacerbated peripheral immune responses to infectious pathogens. Indeed, Sheridan and colleagues showed that when mice are socially disrupted, it can lead to GC resistance. When the GC resistant mice were inoculated with influenza virus (intranasally) they were more likely to die as a result of increased inflammation, lung cellularity and pulmonary consolidation compared to influenza treated GC sensitive mice. In the same study, mice that were exposed to chronic/repeated restraint stress (16 h per day/4 days in a 50 mL conical tube) did not show GC resistance, indicating the perception of stress may have an impact on its physiological outcomes (36). The explanation posited for the GC resistance induced by the chronic stress of social disruption and not restraint is that with social stress, social hierarchy plays a role in the severity of GC resistance and the potential for wounding may modify the outcomes of social stress in order to be able mount an effective immune response (35-36). Taken together, the effects of chronic stress on resistance to GCs may lend evidence to the exacerbated inflammatory responses observed in depressed individuals, who often report high levels of stress (37).

IV NEUROIMMUNE AND BEHAVIORAL EFFECTS OF IONIZING RADIATION EXPOSURE

A) IONIZING RADIATION FUNDAMENTALS

Non-infectious stimuli are capable of activating the neuroimmune system. This is well established in studies investigating the inflammatory effects hypoxia and ionizing radiation exposure. Ionizing radiation (IR) can activate of the neuroimmune system and inflammatory responses. IR sources include therapeutic (e.g. cancer radiotherapy), space and natural (terrestrial) background. Several different types of radiation exist. The most commonly used in both research and clinical settings are x-rays, gamma rays and protons. X- and gamma-rays are both photon radiation, and deposit their energy relatively close to the surface of a tissue (0.5-4 cm depth). Protons, however, are becoming more popular due to their lack of tissue damage to surrounding (non-targeted) tissues. Protons deposit their energy near the end of their particle track (20-30 cm depth) (38). In biological tissues, units of radiation are represented as absorbed dose, defined as the energy deposited in the tissue. Units representing the absorbed dose of radiation are gray (Gy) and radioactivity absorbed dose (rad). Gray is the SI unit, and is the amount of radiation needed to deposit 1 joule (J) of energy in 1 kg of tissue (J/kg). Rad represents the amount of radiation required to deposit 0.01 J of energy in 1 kg of tissue, therefore 100 rad = 1 Gy (39). IR, both therapeutic and non-therapeutic, can cause irreparable damage to cellular components, especially DNA. Evidence exists indicating that the nucleus is the most radiosensitive organelle within the cell, with doses in the range of 1.5 Gy required to cause lethality, but doses up to 250 Gy delivered to the cytoplasm can have little or no effect (40). Acute radiation syndrome (ARS; also known as radiation toxicity or radiation sickness) results

from irradiation of all or most of the body by a high dose of IR over a relatively short time (minutes). The three classic ARS syndromes are, in order of required induction dose (a) bone marrow syndrome (also known as hematopoietic syndrome; requires ~0.7-10 Gy) (b) gastrointestinal syndrome (requires $\geq$ 10 Gy) and (c) cardiovascular/CNS syndrome (requires $\geq$ 50 Gy). Depending on the particular syndrome, symptoms will vary, with increasing severity with increasing dose. ARS is further broken down to 4 stages: (a) prodromal stage (nausea-vomiting-diarrhea (NVD) stage), (b) latent stage, (c) manifest illness stage and (d) recovery or death stage. The prodromal stage can last anywhere from minutes to days, while the latent and manifest illness stage last between hours and weeks, and those exposed to IR who don't recover typically die within several months (41-42). High dose rate (HDR) and low dose rate (LDR) represent how rapidly radiation is delivered, and generally are relative terms to each other. Importantly though, HDR IR is more biologically effective than LDR IR (i.e. severity of ARS increases) (42). IR exposure induces damage on the cellular level through direct and indirect effects. Direct effects result when DNA damage is caused by the direct ionization of DNA molecules by IR. Indirect damage results from radiation's ionization of atoms in the cell, which leave ions and free electrons behind that bind to other molecules in the cell forming reactive species, which are much longer lived than ions (on the magnitude of 10,000x longer). For example, ions and/or free electrons react with oxygen to form reactive oxygen species (ROS) (40). Biological effects of radiation are characterized as either stochastic or deterministic. Stochastic effects (e.g. cancer) are the principle health risk from IR at doses $\leq$ 100 mGy. Deterministic effects (also known as "tissue reactions") result from very high IR doses that predominantly result in cell killing. IR doses accumulated in a protracted manner elicit lesser biological effects than those received in a short period of time (42).

B) NEUROIMMUNE EFFECTS OF IONIZING RADIATION

In terms of neuroimmune effects, it is well established that ionizing radiation leads to production and release of proinflammatory cytokines, adenosine triphosphate (ATP) and generation of reactive oxygen species (ROS) (43-45) . IR is capable of inducing expression and binding activity of nuclear factor κB (NFκB). NFκB is a transcriptional regulator of several gene products, including proinflammatory cytokines (6,43). This data supports other studies showing that IR increases gene expression of proinflammatory cytokines including TNF-α, Il-1β, IL-6 and IL-1RA (43,46-47). The brain is not spared from IR-induced inflammation. Studies have confirmed this, via direct CNS as well as whole body irradiation (48). Direct evidence for neuroimmune system activation by IR is provided by Marquette et al., who showed when rats were partial-body irradiated with shielded heads, IL-1β, TNF-α and IL-6 levels were significantly increased in the brain 6 h post-IR exposure. Similar to a peripheral infection, the vagus nerve was found to be responsible for signaling the peripheral immune status to the brain, and vagotomy prevented the increase in these brain-based proinflammatory cytokines (49).

C) BEHAVIORAL EFFECTS OF IONIZING RADIATION

To date, very few reports exist describing behavioral change following IR exposure. In the 1970s and 80s, Bogo published findings showing that monkeys exposed to 10 Gy IR at dose rates of 0.3, 0.8 and 1.8 Gy/min had impaired performance in memory and cognition tasks in 81% of the 1.8 Gy/min group, but only 7% experienced impairment in the 0.3 Gy/min group. Additionally, as task complexity increased, it resulted in greater behavioral impairment. From these experiments, it was estimated that $\leq$ 3 Gy was the effective dose to induce performance deficits (50). Around the same time, IR exposures of 0.25 Gy were found to induce rodent

conditioned taste aversion (50). More recent behavioral studies have yielded results showing exposure to 3 Gy IR was sufficient to cause a decrease in latency to fall from the rod in a rotorod test, and this dose also caused loss of acoustic startle habituation (51). Lower doses used in another study (0.1, 0.5 or 2 Gy) did not cause significant differences in open field, rotarod or acoustic startle habituation, compared to sham irradiated controls (52). A 5 Gy dose of IR was shown to decrease performance accuracy and reliability in 3 tests of cognitive behavior (psychomotor speed, discrimination accuracy and inhibitory control in a level pressing test) (53).

V NEUROIMMUNE AND BEHAVIORAL EFFECTS OF HYPOXIC EXPOSURE

A) HYPOXIA FUNDAMENTALS

Individuals with asthma, chronic obstructive pulmonary disease (COPD), heart failure, sleep apnea, exposure to high altitude and acute hypotension (as a consequence of stroke or trauma) often experience acute hypoxia (54-58). The brain uses approximately 20% of the body's resting oxygen, but only accounts for about 2% of an individual's body weight (59). Because of its high energetic demand, the brain is exceptionally sensitive to changes in oxygen concentration. In the face of a hypoxic episode, the brain will turn off non-essential brain functions and maintain functions critical for survival. Essential functions, like respiration may undergo a biphasic response, in which the organism initially adapts by attempting to enhance oxygen supply/reduce consumption, before eventually reducing respiratory rate to conserve oxygen, especially in the case of prolonged hypoxia (60-61). In response to hypoxic conditions, blood flow is increased almost two fold to the brain, and is mediated by the brainstem (61).

B) NEUROIMMUNE AND BEHAVIORAL EFFECTS OF ACUTE HYPOXIA

Hypoxia exposure can result in proinflammation (62). Hypoxia causes macrophages to produce proinflammatory cytokines. These proinflammatory cytokines, which are also produced in response to hypoxia/ischemia may be involved in hypoxia-related brain damage. Hypoxia leads to the release of cellular ATP and generation of ROS (63-64). Hematologically, hypoxia leads to increased renal erythropoietin, leading to increased red blood cells (RBCs), thereby increasing oxygen carrying capacity (65). Intermittent hypoxic episodes, such as with obstructive sleep apnea (OSA) patients, express increased levels of inflammatory and oxidative stress molecular markers (66). Mice exposed to a similar intermittent pattern of hypoxia exhibit inflammation, neurodegeneration and deficits in spatial learning (66). Acute hypoxic exposure causes a significant elevation of IL-1β mRNA expression in the brain, which is followed by increased IL-1RA and IL-1R2 2 h later. Acute hypoxia-induced sickness behaviors were shown to be mediated by the upregulated IL-1β, and could be prevented by caspase-1 inhibition and/or IL-1 receptor antagonism, (67). Leptin is a pleiotropic adipokine largely responsible for appetite regulation and energy regulation, and is involved in the recovery from acute hypoxia. In their study, Sherry et al. showed that leptin was required for timely up-regulation of IL-1RA, which is necessary to counter the effects of hypoxia-induced IL-1β (68).

C) NEUROIMMUNE AND BEHAVIORAL EFFECTS OF CHRONIC HYPOXIA

Chronic hypoxia is also related to proinflammation. Very few studies have been conducted investigating chronic hypoxia's direct effects on inducing proinflammation. However, studies utilizing at disease states in which chronic hypoxia is a main component are useful toward uncovering hypoxia-induced proinflammation. Chronic obstructive pulmonary disease

(COPD) is a respiratory condition characterized by chronic hypoxia (69-71). COPD is associated with increased systemic inflammation, indicated by increased IL-6, IL-8 and TNF-α (69-71). Interestingly, there is little information published regarding systemic levels of IL-1β in COPD patients. One study showed IL-1β is potently upregulated and secreted in the carotid body of rats exposed to chronic hypoxia. IL-1β is thought to be involved in altering subsequent vascular response to long-term reduced oxygen (72). No neuroimmune mediated behaviors have been shown to be affected by chronic hypoxia, although there are some reports indicating that OSA, a chronically intermittent hypoxic state, can lead to cognitive impairments. These impairments, however, could be associated with excessive sleepiness, which the authors noted in their study (69). Obesity, to a lesser degree than COPD, is disease associated with low-grade chronic hypoxia. However, while systemic hypoxia may occur, it likely isn't until respiratory function becomes impaired (due to body mass) that true systemic chronic hypoxia takes effect. Chronic hypoxia does occur in obese adipose tissue, which also typically expresses a low level of chronic inflammation as well (73-74).

VI MOUSE TESTING METHODS IN PSYCHONEUROIMMUNOLOGY: AN OVERVIEW OF HOW TO MEASURE SICKNESS, DEPRESSIVE/ANXIETAL, COGNITIVE AND PHYSICAL ACTIVITY BEHAVIORS

A) INTRODUCTION

Since its inception as an interdisciplinary field of science in the 1970's, behavior has been an integral part of psychoneuroimmunology (PNI). Indeed, PNI is generally defined as the study of the interactions between behavior, neural, immune and endocrine system functions (75-

76). Behavior can be, and is largely, used to assess whether a particular stimuli or experimental treatment has the potential to activate the neuroimmune system (75-76). An important concept to recognize in PNI is the bi-directionality that exists between the nervous system and the immune system (76). This is to say that both neural-to-immune and immune-to-neural signaling can and do occur. Shared pathways exist between the nervous and immune systems that use a repertoire of signaling molecules such as cytokines and neurotransmitters (77) that are capable of interacting with both immune and nervous system cells. These bioactives can convey the state of peripheral immunity to the neuroimmune system, communicate the status of neuroimmunity to the peripheral immune system (14,77-78) and provide immuno-activating and deactivating signals to immune cells throughout the body (6).

Observation of innate immune-mediated behavioral change (immunobehaviors) is largely used as a method of measuring neuroimmune activation in response to pathogenic insult of infectious (6) or non-infectious (67) etiology. Sickness behavior, in a classical sense, is a set of coordinated behavioral changes in response to immune stimulation aimed at conserving and redirecting body energy stores toward combating illness and promoting recovery (7). Immunobehaviors are best known for their manifestation in association with bacterial infection (7), but materialize in spectrum of conditions and diseases including cancer (7), autoimmune disorders (79), wounding (80), depression (2) and obesity (81). In any circumstance in which the innate immune system is activated, peripheral inflammatory mediators can impact the brain, altering normal function and causing symptoms of illness/loss of well-being (7,14). Typical sickness behavior symptoms include reduction in food intake, lethargy, malaise, loss of interest in social and/or environmental surroundings, changes in sleep patterns and impaired cognition (2,7,14). Furthermore, continued or dysregulated activation of the neuroimmune system can

progress beyond acute sickness symptoms and transition to behaviors observed in the anxious or depressed (2). Fatigue is often a lingering complication of neuroimmune activation (6), and can present as purely mental or physical or (most commonly) in a combinational form (82). In rodents, exercise behaviors like spontaneous wheel running (83) are helping to unravel the complex biology of physical fatigue while tests examining memory formation (learning) and memory recall are being used to explore mental fatigue (84).

Finally, immunobehaviors are a powerful indicator of neuroimmune status offering insight into the pro-inflammatory milieu of the brain. Altered behavior manifests prior to detectable changes in brain-based bioactives and lingers past their resolution. Such conditions indicate that the brain is very sensitive to small perturbations and that traditional chemical bioassays are often not sensitive enough or appropriately targeted to detect brain-based dysfunction at the molecular level. Hence, use of behavioral testing provides highly sensitive and phenomenologically relevant information in regard to brain function but lacks significant specificity from a mechanistic standpoint. This is either due to an evolution-derived paucity of immunobehavioral phenotypes or a current knowledge/technical deficiency in the ability to parse such behaviors into a multitude of biologically relevant subsets. In this review, methods for measuring sickness, depressive/anxietal, cognitive and physical activity behaviors in mice are described. The tests were chosen based on common usage and validated outcomes.

B) PRE-EXPERIMENTAL CONSIDERATIONS

Behavior is a valuable tool for gauging the presence, severity and duration of innate immune activation. Prior to behavioral experimentation, preparatory procedures are required so that meaningful and repeatable results can be obtained. Mice, like most animals used for

laboratory research, are responsive to the environment. Consistency and reproducibility of results as in any field of science is dependent on the decisions made and precautions taken before initiation of testing. While the following does not account for every possible pre-experimental housing and husbandry scenario, it does seek to articulate and define significant areas of pre-experimental bias. Every animal facility, like every laboratory, is unique with differences obvious and subtle. Standardizing and controlling for clear confounds related to mouse strain, gender and scientific model; and maintaining housing and husbandry practices that support animal behavioral well-being and physical health are key. In addition, the following is not intended to be an absolute guide for "correct" mouse immunobehavioral experimentation, for it is critical that the investigator identify, develop and hone best practice related to the particular area of study with a firm eye on federal laboratory animal regulations and local institutional rules and guidelines.

1. Model and strain choice

A first consideration should be the animal model and strain chosen. The vast majority of PNI behavioral testing utilizes rats and mice. Porcine models have been used (85), as have other types of rodents, especially prairie voles (86). Mice are especially useful in neuroimmune and immunobehavioral research due to their ability to reproduce and mature rapidly and the relative ease to which genetic modification can be applied through mutational, transgenic and knockout approaches (87). Different behavioral phenotypes exist between strains. Therefore, it is important to be aware of and control for potential inter-strain and inter-substrain variances (87), as well as intra-strain variation between mice raised/housed by disparate commercial venders and institutional facilities. Furthermore, genetically altered/modified mice are especially prone to

immunobehavioral alteration and should be selected with such in mind. With genetically altered/modified mice, diligent baseline testing in comparison to wild type animals is essential to ensure the behavior noted is causative (i.e. due to knockout of a specific target gene) and not a result of an unexpected consequence (e.g. saccharin preference anomalies in leptin unresponsive mice (db/db and ob/ob) related to the importance of leptin to sweet taste and not due to a type 2 diabetes phenotype (88)).

2. Gender

Depending on strain, genetic alteration/modification and behavioral test, male and female mice will perform differently. For example, in the elevated plus maze (EPM) females exhibit less general activity (89). When female mice are compared to male mice in a freely explorable open field arena, female mice are less active, are less willing to leave their home cage to enter the open field and are less likely to explore the open field (90). Female mice generally run for shorter distances in a voluntary wheel running paradigm than male mice (91), and will run different distances depending on their current state in the oestrus cycle (92). Thus, regardless of identical housing and husbandry practices, care must be taken in mixing genders during behavioral testing. Such care can be especially frustrating when using genetically altered/modified animals due to in-house breeding deficiencies and difficulty in acquiring adequate numbers of similarly aged animals from commercial suppliers.

3. Age

Natural aging effects decreases in immune functioning with individual variation in severity depending on factors such as life-long physiological stressor exposure (93). It comes

with little surprise then, that neuroimmune-based behavior can also be affected by age. While sickness behavior appears beneficial to young mice, it may be maladaptive in older mice (94). Age can also negatively impact physical activity where aged mice run less (95). Young mice present difficulty in running analyses as well due to progressive increases in distance traveled (96). Investigators should be aware of potential differences arising from non-age-matched experimental mice and older mice reared under disparate conditions.

4. Transportation

Environmental factors play a significant role in how mice behave and respond to neuroimmune stimuli and immunobehavioral treatments. Mice should be allowed a transition phase to acclimatize to a new environment. Whether this is the procedure experimentation room itself (97-98), or the housing room following arrival from a commercial supplier or other outside source (97,99). Biochemically, transportation stress increases plasma corticosterone levels in mice regardless of transport duration, and up to 48 h of acclimation is required for corticosterone to return to pre-transportation values (98). Behavioral change in response to transport has been shown to persist for 4 days (29), and body weight reduction returns to pre-transport levels within 4 days (97,99). Therefore, a period of 5 days of acclimatization is generally required as a minimum for mice undergoing behavioral testing following off-site transportation (99). A minimum of 24 h of acclimatization time following on-site transportation (i.e. between housing and experimental rooms) should also be utilized (99). These are minimum time recommendations for acclimatization. Longer times may be necessary depending on the type of mice used, the duration of transport and the breadth of difference between initial and relocated

environmental conditions (97,99). As with any laboratory test, validation studies are recommended to confirm pre-experimental choice decisions.

5. Light cycle

Alteration of light cycle has been shown to affect natural murine behavioral patterns (99), as well as neuroimmune behavior (e.g. anxiety) (100). Mice are under control of a genetically-driven circadian clock that serves to regulate physiological and behavioral processes in a diurnal fashion (101). Therefore, it is important to ensure that experimental rooms have a similar light cycle to the housing room. In addition, it is advisable to initiate behavioral experiments at the same time of "day" especially when performing repeat testing. Mice are active at night, and, for the majority of testing, should be tested during the dark cycle. Reverse light cycle housing is beneficial so as not to put undue burden on personnel performing the behavioral tests. Mice also are crepuscular (102), with heightened activity during the early (dusk) and late (dawn) components of the light cycle dark phase and should be tested during these peak activity times. Methods for determining the timing of these active periods are described in the locomotor activity section.

6. Temperature/Humidity

Temperature should be largely similar across animal housing facilities, as it is federally regulated by the Office of Laboratory Animal Welfare (OLAW) in the United States (103). According to the Guide for the Care and Use of Laboratory Animals, mice should be housed in a room/environment with temperatures ranging from 68-79 °F (20-26 °C) (34). Mice show neuroimmune and behavioral sensitivity to both heat- (104) and cold-stress (105) indicating the

potential for altered behaviors with temperature fluctuations. Relative humidity should also be maintained at similar levels across animal housing facilities, and the Guide for the Care and Use of Laboratory Animals indicates a range of 30-70%. Cage style, construction, bedding and enrichment materials as well as housing density affect temperature and humidity within the cage microenvironment (103). It is therefore important to recognize that room temperature and humidity may not necessarily reflect intra-cage temperature and humidity, depending on housing factors.

7. Noise

Noise also has the potential to activate neuroimmune signaling pathways and alter behavior, as bell ringing (106) and noise produced by vacuuming (107) have been shown to stress laboratory mice as evidenced by activation of the hypothalamic-pituitary-adrenal (HPA) axis (9,108). White noise generators that create a constant background have been shown to reduce behavioral response to sudden loud noise (109).

8. Odors

Mice use odors for communication, marking territory and in individual and group recognition signaling (99). In addition to using patterns of urine deposition for communication mice also produce specialized odors via several glandular secretions (99). There is also evidence that neuroimmune activation can alter odor production and that odors can induce behavioral change. When mice are administered the classical neuroimmune activator lipopolysaccharide (LPS) they generate olfactory cues to indicate that they may have a transmissible pathogen causing healthy cage mates to socially withdraw from the sick mouse (110). This phenomenon is

also seen in healthy mice housed with tumor-bearing cage mates (111). Finally, exposure to foreign/strange odors (e.g. human associated odors) can result in stress responses (99). Unfortunately, no specific research has been performed examining the duration of olfactory stress responses in mice, nor is there an identified acclimation or exhaustion time for evocative scents. It should also go without mention that eliminating as many olfactory cues within rooms, cages and on experimental apparatuses (e.g. through use of 70% v/v ethanol) is best practice. Mouse handler odors (i.e. perfumes and predator scents (e.g. feline)) should be minimized and/or eliminated.

9. Handling

Physical handling of mice is a well studied modifier of mouse physiology and behavior (112). Therefore, it is advisable to handle mice at least daily so as to acclimate them to their human researchers and to physical contact. With this, mice will be more likely to appropriately and consistently respond to experimental treatment and have a reduced opportunity to succumb to handling stress which can elevate blood corticosterone (113).The handling method used, however, appears important. Mice respond to handling more readily when removed from their cage passively, such as with a tube or cupped hands, as opposed to the more traditional removal by grasping the base of the tail with the thumb and forefinger or soft forceps (114). Hurst and West noted that mice develop a consistent response (measured as voluntary interaction with handlers) by the ninth day of single 60 s handling sessions, regardless whether the mice were picked up by grasping the tail base or allowed to enter a tube or cupped hands before handling (114). Of note, mice picked up by the base of the tail had a lower level of voluntary interaction, compared to tube and cupping methods (114). Furthermore, when investigators acclimated mice

by removing mice from their cages with tubes or cupped hands, the mice were not aversive to scruff restraint, whereas those removed via tail base grasping showed increased distress when scruffed (114). As such, a period of at least 9-10 days of daily handling appears sufficient to ensure a consistent and non-aversive response in mice. If injections are a necessary component of a behavioral experiment, a passive method of mouse cage removal appears best.

10. Housing method/environmental enrichment

Mice are social animals and should be housed as often as possible with other mice (103). Several studies have investigated the impact that individual housing/social isolation has on neuroimmunity and immunobehaviors, and social isolation induces aggression in male mice (115). Individually housed male mice also appear more prone to developing anxiety- and depressive-like behaviors following exposure to unpredictable chronic mild stress (116) despite their increased propensity to explore (90,98). Group housed mice order themselves into a social hierarchy, with 1-2 dominant mice and several subordinate mice. Subordinates as well as dominant mice show similar exploratory levels in an open field context (90) but this seems dependent on the relatedness of the group-housed mice, for an introduced non-sibling dominant intruder mouse evokes social stress and immune cell glucocorticoid resistance in the group-housed mice (35). Therefore, housing mice in groups at a density of one 25-30 g mouse per 77.4-96.7 cm^2 of cage floor area by 12.7 cm of cage ceiling height appears advantageous (103). Interestingly, a clean cage environment (99,117) and novel cage construction materials can reduce mouse welfare and induce aberrant behaviors (99,103). Introduction to a clean/novel cage has been shown to increase plasma corticosterone in mice and to increase physical activity within the first 24 h of exposure. Metal cages, as opposed to more commonly used plastic cages (103),

are colder to the touch, more conducive to noise generation and less permeable to light (99,103). Additionally, the use of solid flooring with absorbent bedding is recommended by most institutional care and use committees, as well as federal regulatory bodies, because wire mesh flooring can lead to paw injury (103) that can confound behavioral experiments involving the innate immune system (6).

Environmental enrichment is thought to enhance mouse well-being by providing motor and sensory stimulation. Environmental enrichment may include nesting material, structures and/or shelters within the cage (103). Lack of environmental enrichment dampens mouse reactivity and alertness in many behavioral tests (118). Environmental enrichment is, however, somewhat strain-dependent because the loss of reactivity and alertness noted above was observed in BALB/c mice but not seen in C57BL/6 mice. In fact, Van de Weerd et al. concluded that male BALB/c mice housed in enriched environments were anxious (118). Olsson and Dahlborn noted simply changing the barren cage environment by placing objects within it does not necessarily lead to "enrichment" (119). Instead, it is important to observe what, how and when behavioral and physiological changes occur in the animal, and if these changes result in long-term improved health and well being of the animal. Some "environmental enrichers" are felt to result in increased stress and anxiety (118,119). Thus, the environment within the cage may be as important as the environment in which the cage is housed in when establishing appropriate pre-experimental procedures.

C) SICKNESS BEHAVIORS

Sickness behavior is classically defined as the non-specific set of symptoms associated with the body's response to innate immune challenge (6). Symptoms of sickness behavior

include anorexia, fatigue, malaise, reduced locomotor activity, loss of interest in environmental and social surroundings and disappearance of body-care activity (6,120). These changes occur in response to brain-based increases in the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), resulting in changes in the motivational state of an organism (2,6). Sick individuals also experience psychological symptoms such as anxiety and depression, and cognitive deficits such as learning and memory loss (discussed in later sections). Frequently used methods for quantifying sickness behavior include social exploratory activity (121), locomotor activity (14,122), food disappearance (14,122) and rotarod testing (51,123).

1. Social exploration of a novel juvenile

The paradigm of social exploration appears to have evolved from work done by Thor and Holloway showing that adult laboratory rats actively investigate and form social memories of conspecifics (124), principally via ano-genital sniffing, nosing, mutual grooming and close following (125). Rodents also do not show behavioral habituation during social investigation provided the conspecific juvenile is novel at each presentation into the home cage of the adult (126). Furthermore, adult male rats do not exhibit aggression towards prepubertal male juveniles but do toward un-related postpubertal males, which is a function of androgen-related odors from the postpubertal rat eliciting aggressive attacks from the adult due to infamiliarity (124). It is this basis in social recognition that first allowed Dantzer, et al. to show that social memory could be modulated by neurohypophyseal peptides (121). This is likely the first experiment that used social exploration as a tool to measure the effects of neuro-active compounds. Social exploratory behavior was adapted from social memory testing by using a different juvenile at each observation time point (127), and due to the advantage of the lack of habituation when using a

novel juvenile, social exploration has been routinely used as a sensitive test of immunobehavioral perturbation. Bluthé, et al. adapted the rat-based Dantzer procedure to mice and since the test has remained largely unchanged (125).

a. Procedure

All observations should be made during the dark/active cycle of the mice. Mice should be housed individually at least 24 h prior to the initial measurement and be allowed to acclimatize to the procedure room (if procedure room differs from housing room) for the time period in which they are individually housed. In some studies, infrared light is used with the aid of infrared- or nightvision-capable cameras (125,127), but use of red-tinted lighting is also acceptable, as mice only have limited ability to detect light from the red portion of the visible spectra (103). All observations of adult-juvenile interaction are video recorded during social exploration testing for later analysis. Within each observation/recording session, each adult mouse to be experimentally observed (subject mouse) is only exposed to a juvenile (challenge mouse) once. Social exploration is initially measured immediately prior to any experimental treatments so as to serve as a baseline of social exploratory activity for each subject mouse. Social exploration is subsequently measured at specific times following experimental treatment, usually every 2 h for the first 12 h post-treatment, and then every 12 thereafter until recovery is reached (125,127). A test session/observation time point consists of introducing a novel challenge mouse (conspecific juvenile mouse of the same sex) into the home cage of the subject mouse for 5 minutes before returning the juvenile to its own home cage (125,127-128). In some instances, the juvenile challenge mouse is housed in a clean 7.62 cm x 7.62 cm x 7.62 cm wire mesh enclosure (baby cage) when introduced into the home cage of the subject mouse (68) to avoid undesired

aggressiveness seen with certain mouse strains like C57BL/6 mice. If a baby cage is used, it is best to acclimate subject mice to the wire mesh enclosure by including it in their home cage during single housing. Once the experiment is completed, the duration (in s) of exploration or investigation of the challenge mouse by the subject mouse is recorded from analysis of the video record (68,125,127). Preferably, analysis of the video record should utilize automated video tracking software (129) such as that developed by Noldus Information Technology (Leesburg, VA). In general, tracking software eliminates observer bias and provides more consistent results. When using tracking software, care must be given as to mouse color versus background color so that the animal is easily distinguished. If tracking software is not available, a trained observer blinded to the treatment groups (blinded trained observer) can manually quantify social exploration without significant prejudice (125,127). Observer training for all video review behavior testing is best accomplished by having personnel new to scoring a behavioral test evaluate previously analyzed videos that have been scored and validated (proficiency testing). When the scoring skills of the trainee observer are within plus/minus 10% of a novel validated video in three consecutive evaluations of novel validated videos, the observer trainee is deemed proficient. This same training should be followed when educating personnel to the use of automated tracking software. During manual video review the blinded trained observer uses a stopwatch to record the time of interaction initiated by the subject mouse with the challenge mouse throughout the 5 min designated investigational period. Social exploration is considered to be subject mouse-to-challenge mouse investigation (not the opposite), including ano-genital sniffing, nosing, following and grooming. With use of a baby cage, nose-to-cage contact is considered exploration. Social exploration is typically shown in graphical display using either

raw seconds of exploration at each time point (125) or as percent control or percent baseline (68).

2. Locomotor activity

As lethargy is a core symptom of sickness behavior, locomotion (120) can be used as a technically easy and high through-put measure of sickness behavior. Spontaneous locomotor activity is advantageous in that it can be assessed without moving mice from their home cage, and automated video tracking software can be easily used for analysis. In addition, wide screen video capture allows for up to 8 mice to be observed at once. Alternatively, specifically designed activity chambers (Versamax from AccuScan Instruments, Columbus, OH) with built-in infrared beam detection systems can be used (130) allowing for real-time analysis. Such testing platforms, however, introduce an element of novel environment and need to be thoroughly cleaned between each mouse tested.

a. Procedure

All observations should be made during the dark/active cycle of the mice. Mice should be housed individually at least 24 h prior to the initial measurement and be allowed to acclimatize to the procedure room (if procedure room differs from the housing room) for the time period in which they are individually housed. As with social exploration, infrared or red lights can be used to provide illumination. At each time point of interest, mouse movement is recorded for 5 min (81), with a camera placed over the center of a single cage or grouping of cages. If multiple mice are being recorded during a given observation point, they should be shielded from one another's view. Movement including total distance traveled, velocity and time spent moving is best

determined from the video record using automated tracking software. However, if tracking software is not available videos can be hand scored by a blinded trained observer. To score manually, a thin-line grid comprised of 6 equally sized rectangles is affixed to the television or monitor screen directly over the cage and the blinded trained observer counts the number of times the mouse crosses a line (line-crossing) throughout the 5 min designated investigational period. A mouse is only considered to have line-crossed if both fore-and hind limbs cross a line (128).

A more powerful method for assessing mouse locomotor activity is through long duration (hrs-days) tracking. This is required when detailing mouse crepuscular movement. While video recording can be used for such evaluation, the data collection, storage and interpretation can be burdensome-to-prohibitive due to video file sizes. Therefore, the use of biotelemetry is the preferred method for this type of testing (131). With this method, a biotelemetric emitter is surgically implanted within the peritoneum of a mouse and a receiver pad linked to a PC running data collection software is placed directly underneath the home cage of the implanted mouse (Mini Mitter, Bend, OR). Mouse movement is tracked and recorded automatically. Specific procedures and training for this method should be provided by the manufacture of the device chosen.

3. Food consumption

Food consumption, (or disappearance, as discussed below), is an indicator of sickness, as individuals experiencing illness often exhibit anorexia. Food consumption gives an indication of whether anorexia is present, which could further be used to indicate if sickness behavior is occurring (14,122). Food consumption can be measured in at least two different ways, as

outlined below. The first uses “food disappearance,” which is often interpreted/estimated as food consumption.

a. Food disappearance procedure

Mice should be individually housed as per social exploration at least 24 h prior to experimentation. With single housing, food should be moved from the overhead cage food hopper and placed in a 8 cm diameter x 5 cm stainless steel bowl in the cage. Steel is preferred over ceramic because ceramic containers can absorb water if they are not completely glazed. In addition, steel dishes can be magnetically secured to the cage bottom or side with the use of a strong magnet. This prevents mice from tipping the bowl and spilling food which can easily occur with plastic bowls. After the acclimation period, and just prior to initiation of testing, new food should be added to the steel bowl and the bowl weighed. This process should occur at the very beginning of the dark/active cycle of the mice in order not to disturb mice during their sleep cycle. Food disappearance is measured by weighing the bowl plus food at fixed intervals, such as every 24 h. For longer term experiments, food can be re-added to the bowl and reweighed (122). The term food disappearance is used in place of food consumption because not all food is ingested. Some food inadvertently falls in the cage bedding (132).

b. Food consumption procedure

Mice should be individually housed as per social exploration at least 24 h prior to experimentation. 24 h prior to testing mice should be fasted but allowed full access to water. An empty 8 cm diameter x 5 cm stainless steel food bowl should be present in the cage. 1 hr prior to testing, mice should be removed to similarly sized cage without bedding but with full water

access. Testing is initiated by wiping the bottom of the bedding-less cage clean and placing a pre-weighed bowl with food in the cage. After 1 hr the food bowl is removed and weighed as are any food remnants within the cage. The difference between food bowl food disappearance and food collected from the cage floor is considered food consumed (130).

A more powerful method for assessing food disappearance and/or consumption is through use of automated food and water intake measurement systems where food and/or water intake initiated by the animal is evaluated by computer controlled electronics (BioDAQ, New Brunswick, NJ). Specific procedures and training for this method should be provided by the manufacture of the device chosen.

4. Rotarod testing

Inducers of neuroimmune activation and sickness behavior impair motor coordination and induce physical fatigue (123). The rotarod performance test can measure motor coordination (5157) by assessing how well mice avoid falling of a rotating rod (133). Some strains of mice progressively perform better on the rotarod test during repeated trials at the same rotational speed indicating a physical training or memory component to this procedure (134-135). Rotarod apparatuses are available via commercial vendors such as AccuScan Instruments (Columbus OH), with some variance in features (number of lanes and/or rod diameter for example). In general, rotarod apparatuses have the same basic design featuring a 3-9 cm diameter rod (51,134) partitioned by plastic divider discs spaced evenly longitudinally along the rod,. The end point measured is latency to fall from the rod (136). Fall detection ranges from pressure-sensitive pads located under the rod to infrared beams that automatically stop an integrated timer when hit or

blocked, respectively. Rotarod performance can, however, be assessed manually from a video record (136) by a blinded trained observer or by automated tracking software.

a. Procedure

Single-housing prior to testing is not required but, like with social exploration, mice require at least 24 h of acclimation to the procedure room. Use of pre-experimentation acclimation to the rotarod is not agreed upon. Some have exposed (trained) mice to the rotating rod, by placing mice on the rod at a low speed (4 rpm (123) and 18 rpm (51)), while others have not (134). Pre-experimentation exposure to the rod has been done 1 week in advance of testing (51) and immediately prior to testing (123). Finally, the test itself can be performed in 1 of 2 ways. The rotarod performance test measures the duration of time a mouse can remain on the rotating rod at a single or several fixed speeds (136). The accelerating rotarod performance test measures the maximum speed of rotation the mouse can tolerate before it falls from the rod in a fixed amount of time (134,136). All pre-test conditioning and testing should be made during the dark/active cycle of the mice (123), although testing has been performed during the light/inactive cycle as well (51).

b. Rotarod performance test

Rotarods should be calibrated such they rotate at a constant speed, and should be kept clean and as odor free as possible between trials, as urine and feces on the rod can affect performance (136). Testing is initiated by placing mice on the rotating rotarod which rotates at a fixed speed. Mice are allowed to maintain themselves on the rod as long as they can and the test session continues until they fall or a designated time point is achieved such as 1-5 min on the

rod. At such time the latency to fall is recorded. Fixed speed trials are best used after significant validation testing on the strain of mouse chosen and are best used on mice with significant loss of coordination because small losses of coordination may not manifest at the speed or time chosen. Some testing protocols investigate several different speeds increasing with each trial. An example of increasing speeds used is 5, 8, 15, 20, 24, 31, 33 and 44 rpm (136). Mouse rest time between increasing speed trials ranges from 10-60 min (134,136).

c. Accelerating rotarod performance test

Pre-conditions are similar as for non-accelerating rotarod testing. This test differs in that the rod accelerates at a constant rate through some specified range of speed (4 to 40 rpm) over a fixed amount of time (5 min). Mice remain on the rod for as long as they can and speed of the rod at the time of falling is the recorded end point (134).

For both methods of rotarod testing, motor learning can be assessed by performing daily repeated trials to determine if mouse time spent on the rod (fixed rod speed) or rod speed endured (accelerating rod speed) improves from trial to trial (134).

D) DEPRESSIVE/ANXIETY-LIKE BEHAVIORS

Depression and anxiety are well known consequences of neuroimmune activation (2,130,137). However, the difficulty in assessing and distinguishing depressive/anxiety-like responses to conditions or experimental treatments lies in the fact that sickness behavior symptoms can overlap with depressive/anxiety-like behaviors. Sickness-induced reduced locomotion is a key confound in that most tests designed to measure depressive/anxiety-like behaviors require mouse movement (2). For this reason, behavioral testing for

depressive/anxiety-like behaviors following exogenous activation of the neuroimmune system should be performed only after overt physical symptoms of sickness have subsided and spontaneous locomotion has returned to pre-treatment levels. Importantly, the presence of depressive/anxiety-like behaviors should be confirmed using anti-depressive and/or anxiolytic therapies to improve/resolve the identified depressed or anxious behavior (2). Tests for depressive/anxiety-like behaviors include burrowing (81,138), the elevated plus maze (139), the open field test (140-141), the zero maze test (134), the tail suspension test (2), sucrose/saccharin preference test (2) and the forced swim (aka Porsolt) test (2). The forced swim test is the test best validated for depression due to its responsiveness to anti-depressives (142). However, investigators should refrain from using any one single test to definitively measure depressive/anxiety-like behaviors. Such behaviors are best examined using a battery of tests. Unfortunately, there is no ideal combination of tests because confounds are mouse strain/model/gender and experimental treatment specific. As an example, sucrose/saccharin preference testing should not be used in mouse testing where serum leptin is affected due to the impact of leptin levels on sweet taste detection by the mouse tongue (88).

1. Burrowing

Rodents are well known burrowers (138). This behavior is related to tunnel maintenance and possibly defense. Defensive burying is a known indicator of anxiety and can, itself, be measured (138). Burrowing appears to be largely hippocampal-driven but mouse strain differences exist with C57BL/6 mice burrowing more than 129ES2/Sv mice (138). Burrowing is associated with depressive/anxiety-like behavior where reduced burrowing reflects an increased

depressive/anxiety-like state (81). As burrowing utilizes relatively simple equipment and minimal labor, it is a simple and inexpensive method for evaluating immunobehaviors (81,138).

a. Procedure

All observations should be made during the dark/active cycle of the mice. Mice should be individually housed as per social exploration at least 24 h prior to experimentation and, with single housing; a clean empty burrowing tube should be placed in the cage. Burrowing tubes can be constructed from standard white 6.8 cm diameter PVC piping cut to 20 cm in length (138). The open end of the burrowing tube is elevated 3 cm by bolting two 50 mm machine screws 1 cm from the open end, and spaced so that the tube entrance is elevated. This elevation keeps burrowing substrate from spilling out of the open end. The closed end is sealed with a standard PVC end cap (81). Testing should begin 3 h prior to the onset of the dark/active phase of the light cycle and is initiated with addition of burrowing substrate to the tube (81). The burrowing substrate used needs to be suitable to the mouse strain and experimental treatment. Pelleted mouse chow, gravel or sand are common materials used for burrowing (138). The burrowing tube can be completely filled (138) or filled with a fixed amount of substrate (81) if ceiling effects are not a concern. Ceiling effects arise with vigorous borrowers. These mice will remove all substrate from a tube in a rapid time frame obscuring any difference in burrowing activity relative to time. After substrate is placed in the burrow, the burrow plus substrate is weighed and returned to the cage. If mouse chow is used as a substrate, food from the cage food hopper should be removed for the duration of the burrowing test (138). Depending on anticipated mouse burrowing activity, experimental observation time points can range from 1-24 hrs. Amount burrowed (in grams) is calculated from the pre- and post-burrowing weight of the tube plus

substrate. Following a measurement the burrowing tube can be refilled, reweighed and returned to the cage for additional testing (138). Alternatively, a single measurement of burrowing can be utilized (81). Occasionally, with poorly burrowing mice, one or several training sessions may be necessary, and a practice run with the mice to be used in the experiment can, and has been shown to, improve burrowing activity and reduce variability between animals (138).

2. Elevated plus maze

The elevated plus maze (EPM) is a simple method to measure anxiety-like behavior in mice. Anxiety is assessed by comparing the time spent in the open (exposed) versus closed (walled) arms of a 4-arm radial maze (139). The advantage to the elevated plus maze is that it eschews use of noxious stimuli like foot shock, food/water deprivation and/or loud noise. Instead, it relies on the predilection of mice to favor dark enclosed spaces over open and obviously elevated environments (139). Accommodation and/or learning can occur with repeated exposures to the maze. Therefore, EPM is generally administered as a single exposure with control mice for comparison (139).

a. Procedure

All observations should be made during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. The maze can be made of a variety of materials but those that can be easily wiped clean between each mouse tested like stainless steel or plastic are recommended. Maze shape is that of a plus sign where the four arms are spaced 90° apart, radiating from an open central 5 cm x 5 cm platform. Arm length and width are 25 cm x 5 cm,

respectively. Maze elevation should be at least 40 cm from the floor (139,143). Arm wall height is 15 cm in the "closed" arms and there are no side walls in the "open" arms. The central 5 cm x 5 cm platform has no walls. The open arms are at 180° from each other, likewise with the closed arms. Unlike the tests described above, EPM should be well lit by overhead white light. Significant arm wall-generated shadows, especially those confined to a single arm, should be avoided. Testing is initiated by placing the mouse in the open central 5 cm x 5 cm platform. Each subject mouse needs to be introduced to the maze in a similar fashion and placed on the maze in the same spot with analogous orientation (139). Mouse exploration is video recorded for 5 min (139) to 10 min (144). Time spent in open and closed arms, the number of entries between arms, (defined as all 4 paws of the mouse crossing the threshold of an arm), frequency of head-dips (downward movement of the mouse head toward the floor from an open arm), rears and stretch-attend postures (139) are best determined from the video record using automated tracking software (128). If tracking software is not available videos can be hand scored by a blinded trained observer (139).

3. Open field test

The open field test (OFT) can be used to measure movement (141) and anxiety-like behavior (140-141). OFT apparatuses are walled arenas that vary in shape (square, rectangle, circle) and size (250-2500 cm^2) (141). OFT testing should not be used as a surrogate test for spontaneous locomotor activity because the OFT uses a novel environment (141). Anxiety-like behavior in the OFT is evaluated by examining mouse movement throughout the arena with a special focus on the amount of time the mouse spends/moves next to walls of the OFT apparatus (thigmotaxis). The novelty, size and white light illumination of the OFT contribute to

anxiogenesis (141). Procedures vary considerably but the open field arena is usually brightly lit in studies investigating anxiety (140).

a. Procedure

All observations should be made during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. For testing of anxiety-like behaviors the arena should be larger than 1600 cm^2 (141). Arena wall height should be at least 35 cm so as to limit the ability of the mouse to see over/above the arena. The arena can be made of a variety of materials but those that can be easily wiped clean between each mouse tested, like stainless steel or plastic, are recommended. Testing is initiated by placing the mouse in the center of the arena, and each subject mouse needs to be introduced to the arena in a similar fashion and placed in the same spot with analogous orientation. Movement through the arena is video recorded for 5-10 min and analysis of movement is best documented with automated tracking software because thigmotaxis is easily appreciated with this method. Path tracing is a key aid in that overall patterns of movement can be evaluated. Such patterns supplement the usual measurements of time spent adjacent to the arena walls, wall preferences, time spent not adjacent to the arena walls, overall distance traveled, velocity and time spent moving. Videos can be manually examined using a line-crossing scoring approach (similar to that described for spontaneous locomotor activity) but this method should be carefully validated due to the complex grid pattern needed to ascertain time spent close to the arena walls. Due to this intricacy, some have used the end point of total distance moved plus time spent in the central 25% of the arena (143). Finally, OFT has been used as a repeated measure to determine if therapeutics improve performance over time (145).

4. Zero maze

Like the EPM, the zero maze measures anxiety-like behaviors in mice (134) by using elevation and open and closed areas. Therefore, time spent in the open indicates a reduced level of fear/anxiety as demonstrated by use of anxiolytic agents and their ability to increase time spent in the open area of the zero maze (134). The advantage of the zero maze over the EPM is the elimination of the central platform of EPMs, which can complicate analysis of open/closed arm comparisons (146).

a. Procedure

All observations should be made during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. Maze design varies but in general is comprised of a circular track 30-45 cm in diameter that is 3-5 cm wide. Maze elevation should be at least 40 cm from the floor (134,147). The track should be divided into four quadrants with two quadrants having no side walls and two quadrants having side walls at least 15 cm in height. These open and closed areas should alternate. As with all maze constructions, materials that are easily wiped clean between each mouse tested are recommended (134,146-147). Ample but dim (40-60 lux) white lighting should be used to achieve similar illumination of both the open and closed quadrants (146). Testing is initiated by introducing the subject mouse to the middle of a closed quadrant (designated as the starting quadrant). Each subject mouse needs to be introduced to the maze in a similar fashion and placed on the maze in the same place and orientation. Mouse exploration is video recorded for 5 min (146). Time spent in open and walled arms, the number of transitions between open and walled quadrants, number of rears, number of head-dips (the actual dipping of

the head over the edge of the track in an open quadrant), time spent grooming, the number of stretch attend postures and number of fecal boli in each type of quadrant is best determined by using a combination of automated tracking software and direct observation after testing (fecal boli) (134,146). If tracking software is not available videos can be hand scored by a blinded trained observer. Entry into a quadrant occurs when all 4 paws cross the threshold of an open or walled area (146).

5. Tail suspension test

The tail suspension test (TST) is a commonly used behavioral test for assessing depressive-like behavior in mice. It is thought to induce an escape response (148). With increased depressive-like behavior the mouse fails to extricate itself from the apparatus and becomes immobile. Increased immobility indicates a greater degree of depressive symptoms (149). Importantly, antidepressants shorten immmobility offering a degree of validation to the test's usefulness in measuring depressive-like behaviors (148). The TST can be automated through use of commercially available apparatuses that utilize computer-linked linear load cells and load cell filters to determine mouse movement/struggle (Med Associates, St Albans, VT). As with any commercially purchased device, specific procedures and training should be provided by the manufacture of the device chosen. However, certain basic procedures should be followed and considered with use of the TST including the difficulty in examining young (especially C57BL/6) mice due to their robust tail climbing behavior and penchant for extracting themselves from the device.

a. Procedure

Unlike all the previous behavioral tests described the TST should be administered during the light/inactive cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. Testing is initiated by affixing the mouse to the apparatus "hook" with adhesive tape wrapped around the tail at three quarters of its length from base. Hook the mouse to the apparatus through the tape as close to the tail as possible. The tail should remain straight so as not to injure the mouse. Mice should be suspended as uniformly as possible, and if multi-mouse devices are used the mice should be shielded from each other's view (149). Immobility verse movement/struggle should be measured for 6 min. Non-automated devices can be constructed, which are essentially chambers with hooks. Mouse behavior can be video recorded and immobility determined from the video record by automated tracking software (150) or a blinded trained observer (151). With any of the aforementioned analysis techniques, time of immobility is compared between control and experimental groups of mice (149).

6. Forced swim test

The forced swim test (FST), also called the Porsolt test for the investigator who developed the test in rodents, like the TST, is a tool for assessing depressive-like behavior in mice. The FST is relatively easy to administer (152) and felt to be the best validated test for depression by the pharmaceutical industry (153) This test evolved from the observation that rats will develop an immobile posture after an initial attempt to escape from an inescapable cylinder filled with water. FST-induced immobility is thought to represent behavioral despair (failure of persistent escape behavior) or a development of passive behavior that causes the animal to stop

actively coping with a stressor (152). The FST has several disadvantages when compared to the TST. The FST appears to be more stressful for mice and carries a risk of hypothermia (149). Mice of varying fatness are also difficult to assess due to inherent buoyancy differences.

a. Procedure

Like the TST, the FST should be administered during the light/inactive cycle of the mice (153). Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. As with most device requiring tests, equipment design varies. A simple set up is to use clean white or black cylindrical PVC containers 16 cm in diameter and 31 cm in height (essentially 2 gallon open head pails) containing 20 cm of water maintained at 25°C ± 1°C (21). The FST should be performed under 30 lux white light (154). Testing is initiated by introducing the subject mouse to the water-filled container. Mouse swimming is video recorded for 6 min (21). Immobility is determined from the video record from the last 5 min of the FST using either automated tracking software (155) or a blinded trained observer (21). Immobility scoring should not include movements necessary for the mouse to maintain its head above water (149). Like the TST, time of immobility is compared between control and experimental groups of mice.

7. Sucrose/saccharin preference

Anhedonia, or the inability to gain pleasure from otherwise enjoyable experiences, is one of the features of depression (156-157). In mice, their preference for sweetened solutions has been exploited to measure anhedonia. The decreased consumption of a sweet-tasting solution is indicative of anhedonic behavior and can be reversed with anti-depressives (157). Sucrose (157)

and saccharin (81) solutions are commonly used opposite normal tap water in a 2-choice test. For investigators concerned with mouse caloric intake, saccharin is the recommended sweetener (81). Advantages to the sucrose/saccharin preference test is that it can be run continuously for many days without significant concern of adaptation or learning.

a. Procedure

Three days prior to testing mice should be singly housed in standard cages adapted for 2-bottle water access (adaptation phase). If the experimental design requires mice to be challenged with a neuroimmune activator, each bottle should contain either saccharin as a 0.4% sodium saccharin solution (1% for sucrose can substituted for saccharin) or water. If the experimental design does not require exogenous challenge as with a comparison of mice of different strains or genders, the adaptation phase should consist of both bottles being filled with water. The adaption phase is especially important to experiments using endogenous immune activators so as not to elicit the behavior of conditioned aversion where the mouse associates a newly introduced substance like saccharin/sucrose with the cause of their loss of well being (158). After the adaptation phase, mice are usually administered a challenge at the beginning of their dark/active cycle and then returned to the cage in which they were adapted in the presence of both water and saccharin (testing phase). Fluid consumption is recorded every 24 hours. Fluid consumption is determined by bottle weight (81). In order to control for the development of bottle bias, bottle position of water versus sweetened solution should be switched on a regular but defined basis such as halfway through the experiment or every 24 h. Bottle switching should also be practiced during the adaptation phase usually at 24 h intervals. Sweetened solution preference is generally reported as a percentage of sweetened solution consumption/disappearance to total fluid

consumption/disappearance (157). A 50/50 consumption of sweetened solution to water equates to anhedonia but a ratio in which water consumption/disappearance exceeds sweetened solution consumption/disappearance is indicative of aversion and should trigger concerns as to the applicability of the results to the measurement of depressive-like behaviors (81).

E) COGNITIVE BEHAVIORS

Neuroimmune activation can dramatically impact cognitive function (79) causing learning and memory deficits. Most mouse-based behavioral tests for cognitive function make use of memory and focus either on the ability of the mouse to form new memories (memory formation) or recall old memories (memory retention). Memories involving location (spatial memory) are especially utilized (79). A cornucopia of cognitive function tests exist. Some of these tests have been specifically designed to identify specialized aspects of learning and memory such as olfactory memory (159). Given that peripheral innate immune driven neuroimmune activation is relatively brain-region non-specific, cognitive tests that cover more global aspects of brain function are favored by PNI investigators.

1. Novel object recognition

Novel object recognition is a test of working memory in mice. The test exploits the innate tendency of mice to investigate a new entity (160). Novel object testing is one of the simplest of cognition tests, but test variations are described that add significant complexity through mixing of objects, object placement (novel location testing) and testing arena conditions (81,160-162). The setup for novel object recognition typically depends on what sort of memory function a researcher desires to investigate (160). An advantage of novel object testing over other

seemingly more powerful maze-based memory tests is its adaptability to repeated measure testing (160). In essence, as long as the mouse is well adapted to the familiar object, changing out the novel object after each exposure allows for a new round of testing. This feature is very useful when looking at recovery from a neuroimmune challenge.

a. Memory recall procedure

All observations should be made during the dark/active cycle of the mice. Mice should be housed individually at least 24 h prior to the initial measurement. The 24 h training phase is initiated by introducing two identical objects into the home cage (standard shoe-box cage size; 28 cm in length; 17 cm in width; 12.5 cm in height) of the singly-housed mouse. The objects are placed 10 cm apart at the short-side wall end, 5 cm from the short side wall and 3.5 cm from the long-side wall. Tall (3-5 cm in height) complex objects are preferred because when a tall complex object is introduced during the testing phase it provokes significantly more exploration time. Tall complex objects can be constructed from Lego® blocks (Enfield, CT). Magnets can be used to secure the structures to the cage floor. All structures should be taken apart and cleaned prior to reuse. After the 24 h training phase, the mouse is subjected to the chosen neuroimmune activator. At relevant times after the applied immunobehavioral challenge, the memory recall testing phase is initiated by placing the mouse in a home-cage like arena (including bedding) which contains a similar object set up as in the training phase where one of the familiar objects has been replaced by a novel tall complex object. The mouse should be introduced at the cage end opposite the objects. No spatial clues should be present in the testing/training area. Object exploratory behavior is video recorded for 5 min and object investigation is determined from the video record by either automated tracking software or by a blinded trained observer. Object

exploration is considered as contact by mouth, nose or paw. Accidental contact such as bumping into an object while passing should not be considered (161). Mice with a memory recall deficit should examine both the familiar and novel objects equally (163). Once recovered from neuroimmune activation, mice should explore the novel object over the familiar object. This test cannot be performed as a repeated measure and, thus, requires separate groups of mice to determine at what time after neuroimmune activation cognitive recovery occurs.

b. Memory formation procedure

All observations should be made during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. Memory formation testing differs from recall testing in that training occurs after endogenous activation of the neuroimmune system instead of before (160). The procedure is identical to the above except at relevant times after the applied immunobehavioral challenge mice are trained for 1 hr with the two familiar objects in the shoebox-sized testing arena then returned to the home cage. After 1 hr in the home cage, testing is initiated by placing the mouse back in the testing arena where one of the familiar objects has been replaced by a novel object. Recording time and scoring are identical to the above. Mice with a formation deficit should examine both the familiar and novel objects equally. Once recovered from neuroimmune activation, mice should explore the novel object over the familiar object. This test can be performed as a repeated measure as long as the novel object is always new.

2. Fear conditioned learning

Fear conditioned learning is a form of classical (Pavlovian) conditioning where an association between a stimuli and its aversive consequence(s) is made (164). Fear conditioning is a highly conserved behavior that occurs in mice both in the laboratory and in the wild. In PNI research, it is a useful tool for evaluating emotional memory formation and recall (164). Fear conditioned learning is generally a one-trial learning procedure and is unique from other cognitive tests in that the investigator regulates the parameters of the stimulus. Factors that impact mouse hearing, like age, are important to consider prior to use of fear conditioned learning, as auditory sensory decline will negatively affect sound-based contextual cues (164). Like novel object testing, fear conditioned learning can be used to test memory formation and memory recall. For memory formation testing, the training phase (see below) is conducted after neuroimmune stimulation. For memory recall testing, the training phase is conducted prior to neuroimmune stimulation.

a. Cued fear conditioning procedure

All observations should be made during the dark/active cycle of the mice. Mice should be single housed for this behavioral procedure and, like with social exploration, mice need at least 24 h of acclimation to the procedure room. Automated commercially-available fear conditioning apparatuses (San Diego Instruments, San Diego, CA) are the easiest way to adapt this testing paradigm. General apparatus parameters are fairly uniform. There is a shock generator and scrambler that delivers a 0.1-1.0 mA foot shock through a wire grid floor in concert with a sound generator that produces auditory cues, all contained in a shoebox cage-sized chamber (164). It is recommended that sound meters and voltmeters are used to verify and record stimulus intensities

(164). Prior to testing mice require training. In the initial training session, mice are placed in the fear conditioning apparatus for 120 sec (phase A) before the presentation of a 30 sec sound cue (phase B). A 2 sec foot shock is delivered immediately after the sound cue (phase C). Mice are returned to their home cages 30 sec after the shock ends. Repeat training can be utilized to reinforce the memory. As noted above, training relative to neuroimmune stimulation determines whether memory formation or recall is being tested. Testing is usually initiated 24 h post-training and consists of re-introducing the mouse to the fear conditioning apparatus and re-presenting the sound cue. The sound cue now lasts for 180 sec. Mouse behavior during this 180 sec period is recorded with a side-mounted video camera. However, apparatuses with a beam detection grid system linked to a PC can automate analysis. With video recording, freezing and non-freezing behavior is scored by a blinded trained observer at 10 sec intervals. Freezing is considered a complete lack of mouse movement (164). Fear conditioning is presented as number of freezing episodes. With an automated detection system, actual time spent frozen can be determined.

b. Contextual fear conditioned procedure

Contextual fear conditioning uses the same pre-experimental and scoring procedures as for cued fear conditioning. However, in contextual fear conditioning, no sound cues are delivered. The mouse is expected to associate the apparatus with the foot shock. Testing time is 180 sec. Complexity can be added by using an alterable microenvironment within the fear conditioning apparatus (altered contextual fear conditioning). A variety of cues from visual to olfactory can then be utilized (164).

3. Spontaneous alternation

Spontaneous alternation is the simplest spatial memory test to perform in mice. Increases and decreases in spontaneous or perfect alternations reflect improvements and impairments, respectively, in spatial memory function (81,165). Spontaneous alternation can be performed in a variety of maze types including radial arm, T and Y (81,166). Sub-forms of spontaneous alternation testing have also been described including forced-trial alternation, where one arm of the maze is closed off, forcing the mouse to enter the open arm without choice. In a subsequent testing, the closed arm is then made available (166). Interestingly, certain mouse strains have been shown to be biased in their turning direction (166), and this should be considered and controlled for. Like novel object testing, spontaneous alternation can be used to test memory formation and memory recall. For memory formation testing, spontaneous alternation test is conducted after neuroimmune stimulation. For memory recall testing, the mouse is tested in the Y-maze (which serves as a training period), exposed to a neuroimmune activator and then re-tested in the Y-maze. In addition, spontaneous alternation can be performed as a repeated measure and maze performance usually increases with repetition. Repeated measure testing is generally preferred because a "one and done" testing strategy is more indicative of locomotor activity and less dependent on spatial memory (166).

a. Procedure

All observations should be made during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. As with all mazes described previously, the Y-maze used for spontaneous alternation should be made of a material that can be easily wiped clean between

each mouse tested. Clear Plexiglas is preferred so that a different black-colored design (lines, circles or triangles) can be affixed to the outside wall of each arm to provide intra-maze visual cues. The maze base is a opaque blue. Maze shape is 3 equally spaced arms 120° from each other (radial Y). Arm length is 40 cm, arm width is 9 cm and arm wall height is 16 cm (81). Testing is initiated by placing the mouse into the distal end of a randomly chosen arm (as assigned by a random number generator). Each subject mouse needs to be introduced to the maze in a similar fashion and placed on the maze with analogous orientation. Mouse exploration is recorded for 5 min (3 and 15 min have also been used (167)). If the experimental design allows, mice should be tested every 24 h for 4 consecutive days. Perfect alternations are determined from the video record by a blinded trained observer. A perfect alternation is defined as exploration of two different arms of the maze sequentially before a return to the starting arm (e.g. beginning in arm "C," moving to arm "A," then to arm "B," before returning to arm "C" again) (81). Number of "regular" alternations should also be scored. Regular alternations are defined as entering all three arms within a sequence of four arm entries (e.g. ACAB is considered an alternation, whereas ACAC is not) (166). Arm entries occur when all four paws of the mouse pass the threshold of the arm entrance (81). Results are represented as total alternations or perfect alternations divided into the total possible alternations or perfect alternations, respectively (166). Perfect alternation scoring is considered more rigorous.

4. Barnes maze

The Barnes maze, like spontaneous alternation, is a test of spatial memory. This test combines several aspects of the previously mentioned mazes including elevation, open/exposed illuminated space and a dark enclosed area (168). Use of the Barnes maze was popularized as an

alternative to the Morris water maze (described in the next section) because swimming may produce anxiety (169). Removal of water also allows for more balanced testing of mice of different fat density due to elimination of the buoyancy effect. Importantly, the Barnes maze appears to rely on the same hippocampal-dependent memory function as the Morris water maze (168). As with any maze designed to test spatial memory, extra- and intra-maze cues serve as location reference points and without these cues mice perform less well (168). Like spontaneous alternation, the Barnes maze can be used to test memory formation and recall depending on when neuroimmune activation is triggered relative to the training period. However, recall is significantly simpler to measure when using transient memory impairment paradigms.

a. Procedure

All observations should be made during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. As with all mazes, construction materials should be easily cleanable. A typical Barnes maze is a 90 cm diameter white acrylic disc with 12 equally spaced through-and-through holes arranged 5 cm from the outer edge of the disc. Hole diameter is 5 cm and the maze is situated 56 cm off the floor (168). A tunnel like extension attached to an enclosed sealable 8 x 8 x 8 cm chamber (escape chamber) needs to be freely fittable to one of the holes from underneath the maze. Thus, for the mouse to escape the maze, it must enter a hole. Extra maze cues, such as different geometric shapes are placed around the maze and on the walls of the room (169). Prior to testing multi-day training is required. Training occurs 4 times per day (during the dark/active cycle of the mouse) for a 5-day period. In each session, the mouse is introduced to the maze (lit at 1200 lux) via a non transparent holding chamber placed in the

center of the maze. Time spent in the holding chamber is 30 s. After the holding chamber is unsealed, the mouse is allowed to explore the maze freely for 5 min. During each training session, the escape chamber should remain under the same assigned escape hole with all other holes blocked. During the 5 min exploration period, the mouse should find the escape chamber hole. If the mouse fails to find the escape chamber hole, it is picked up and placed near the entrance of the escape chamber hole and allowed to enter. Once the mouse enters the escape chamber the mouse is removed from the maze and the training session is ended (168). One hr after the final training session a probe trial is conducted in which all of the holes are blocked (preventing any escape) and the mouse is allowed to explore the maze for 5 min. Successfully trained mice with functional spatial memory should actively search for the remembered escape chamber hole in the appropriate location. Mouse introduction to the maze during the probe trail uses the holding chamber technique as performed during training. After immunobehavioral stimulation, testing is initiated re-performing a single 5 min training procedure. Mouse behavior is video recorded and maze performance evaluated from the video record using a combination of automated tracking software and observation. The mouse should use the extra-maze visual cues to locate the remembered escape chamber (168). Scoring the trials consists of tallying the frequency of errors committed before entering the escape chamber (examination of the an incorrect hole), timing the latency to find/enter the escape chamber and determining the path length to the escape chamber. Different variations of the Barnes maze exist and include a hidden-target fixed-location modification in which the extra-maze cues were always in the same location, but the maze was rotated (168).

5. Morris water maze

The Morris water maze (MWM) is used for assessing spatial or place learning. Advantages of the MWM include no requirement for pre-training, high reliability across different tank designs and proved validity in measuring hippocampal-dependent spatial and reference memory. Learning impairments in the MWM are independent of locomotor deficits, as locomotor reductions do not seem to affect swim speed (170). The MWM is an open circular pool filled with water. Mice must swim and search for a small, hidden platform just below the surface of the water in a fixed location utilizing extra-maze cues. The maze is constructed of a circular, stainless steel tank 122 cm in diameter, with 51 cm high walls, with non-reflective inner surfaces. Contrasting colors between the inside of the tank and the mouse allow easy integration of automated tracking software for the analysis of swim path, duration and location. As the purpose of the MWM test is to induce the mouse to use distal cues, any seams or recognizable patterns on the inside of the pool are not recommended. The platform (made of a plastic or PVC conduit shaft with a plastic or acrylic platform on top) is typically square or circular in shape and is clear or matching in color to the inside of the swim tank. The platform is positioned just below the water level (0.5-1 cm). Water temperature should range between 24-26 °C (171). The room in which the MWM test takes place in should allow for ample distal visual cues like for the Barnes maze. Studies have shown that lack of cues can negatively affect MWM performance (170). The investigator should be aware that their presence in the procedure room during MWM testing may make them a distal visual cue. All variations of the MWM procedure should take place in an illuminated, albeit indirectly lit room (to avoid reflections or glare on the water surface, which can make scoring with automated tracking software difficult) (170). The maze is divided into four, equally-sized quadrants with the platform positioned in the center of one of

these quadrants (170). The procedures for spatial acquisition and reversal learning are described below but a variety of modifications exist. A key concern in MWM spatial memory testing is that the probe trial should be spaced temporally from the last training session to effectively and reliably measure reference memory formation (170).

a. Spatial acquisition procedure

All testing should take place during the dark/active cycle of the mice. Single-housing prior to testing is not required but, like with social exploration, mice need at least 24 h of acclimation to the procedure room. The mouse is placed (not dropped) at the selected start position in the maze, facing the tank wall. A timer or video tracking should be started immediately at the placement of the mouse in the maze. The timer remains on until the mouse reaches (comes in contact with) the platform. Standard trial limits of 1-2 min per trial are usually used, and mice that have not reached the platform should be placed on or guided to it by the investigator (170). The animal should remain on the platform for 15 sec. This step helps mice orient their position in space relative to the extra-maze cues (170). Following the inter-trial interval, the mouse is again placed in the maze, this time in a different but predetermined location (most protocols start the mice from one of four positions – south (the investigators position), north (opposite the investigators position), east (to the right of the investigators position) and west (to the left of the investigators position)). Trials are repeated 4 times per day for 5 days. Following training, the experimental treatment is administered and time is allowed for the treatment to take effect, or in the case of neuroimmune activators, for sickness behavior to resolve so as not to confound the results (2). The probe trial is run, during which the platform is removed from the maze. The probe trial is video recorded and lasts 60 s, after which the mouse is

removed. The objective of the probe trial is to determine whether or not the mouse can recall memories of where the platform was during training sessions based on the distal visual cues (170). End points measured in spatial acquisition include the number of platform site crossovers, time and distance swam in the target quadrant relative to the other quadrants, time in a predefined radius around the original platform position (larger than the original platform itself), average distance swam to target site and latency to first target site crossover. For investigators without automated tracking capabilities, blinded trained observers should use a timer to calculate the time spent in the aforementioned areas, as distance traveled is not feasible to measure with trained observers. Percent time spent in the target quadrant or percent of distance swam in the target quadrant are the most common reported end points in MWM spatial acquisition testing (170).

b. Spatial reversal testing procedure

Spatial reversal testing determines the ability of the mouse to extinguish a particular memory in favor of forming a new one (170). In this paradigm, training procedures are the same as they were for spatial acquisition, but the probe trial differs. During the reversal training probe trial, the platform is moved, typically to the opposite side of the maze, but cues remain in their same position as during training trials (171). Mice are placed first on the platform for 30 s to allow them to gain some spatial cues as to where the new platform location is. Mice are then given 1-3 trials to reach the platform, starting from different locations if necessary (171). The same end points are used in spatial reversal training as with spatial acquisition (170). Since the platform remains in the maze, latency to reach the platform, swim speed and total distance swam

are also used as end points (171).Some variations of the MWM include repeated learning, latent learning and cued learning (170).

F) PHYSICAL ACTIVITIES

An acute reduction in physical activity is a sign/symptom of sickness and is associated with fatigue (2). Chronic low-grade inflammation is also linked to altered patterns of physical exertion and fatigue (2). Physical activity and fatigability can be measured with techniques adopted from exercise research and include voluntary running wheel and exhaustive/forced running. These tests are more powerful than spontaneous and long duration locomotion testing described earlier in that they can tease out more subtle activity differences. Animals that engage in more spontaneous physical activity generally have less fatigue, higher fitness levels and better performance in forced exercise testing (172). Important behavioral differences appear to exist with spontaneous and forced exercise. Wheel running is spontaneous and thought to be under central nervous system control. The concept of motivation is critical to this behavior and sickness-associated fatigue appears to be a modulating factor (173-174). In contrast, exhaustive exercise, such as forced treadmill running, is generally controlled by muscle and/or cardiovascular limitations (175). The rapidity with which an animal discontinues an exhaustive exercise test may also be governed by immunobehavioral fatigue (175). PNI investigators most often use spontaneous wheel running (SWR) (95) and forced treadmill running (FTR) (173) to probe the impact of immunobehaviors on physical activity. SWR is preferred for the high through-put testing in that it can be remotely monitored with little demand on personnel. FTR is much more labor intensive but allows for considerable customization including alterability of duration, frequency and intensity. FTR is also considerably more stressful to mice.

1. Spontaneous wheel running

A key advantage of SWR is that it can be assessed without moving mice from their home cage and the length of examination time can be very long. A disadvantage is that mice need to be singly housed. Specialized caging is needed to accommodate the running wheel and bedding must be correctly adjusted and monitored so as not to interfere with the wheel. A basic running wheel may measure only revolutions of the wheel and may need manual resetting at each data collection point. Advanced running wheel systems (Mini Mitter, Bend, OR) can obtain hourly, daily or weekly distances run. Regardless of the running wheel sophistication, wheels need to be clean and well lubricated. Running wheel size and structure should also accommodate the size of mouse used. In long duration studies, cage cleaning and contact with animal facility and/or investigative personnel can result in an acute reductions in running. For the below procedure, an automated, multi-channel running wheel system (Mini Mitter, Bend, OR) is utilized. Specific procedures and training for any given wheel should be provided by the manufacture of the device chosen.

a. Procedure

Mice need to be individually housed for experiments with running wheels and need to acclimate to the procedure room for at least 24 h prior to experimentation. Groups of mice in cages with locked running wheels and in cages with no wheel present should be included for proper experimental controls. Prior to experimentation (such as neuroimmune activation), a baseline measurement is recorded in case post-hoc normalization of distance run is required. After immunobehavioral stimulation, mice are immediately returned to their cage and allowed access to the wheel (rotating, locked) or cage environment (no wheel). A 10 day course of wheel

running is recommended. With automated running wheel systems, total distance run is reported. With manual wheels, wheel revolutions are recorded and distance traveled calculated by multiplying the wheel circumference by the number of revolutions. A limitation in SWR is the absence of a running intensity marker. However, some sophisticated wheel systems can record revolutions/min providing some insight into intensity.

2. Forced treadmill running

FTR better measures mouse fatigue (175). Like running wheel systems, mouse treadmills vary in sophistication with some allowing both uphill and downhill running (IITC Inc. Life Science, Woodland Hills, CA). Treadmills coupled to oxygen consumption systems can be used to determine mouse "fitness". Non-rodent treadmills (Jog-A-Dog, Ottawa Lake, MI) divided into lanes with Plexiglas dividers allow for high through-put studies of up to 20 mice. Treadmills should contain a protective end (foam) to prevent mice from being thrown from the device and to provide an impetus to move forward should the mouse reduce its speed or stop running. Mice will respond to the contact of the tail/hind portion with the protective end. A ventilated cover is also recommended. Intra-experimental prodding can be used if a mouse or mice appear to predominantly "ride" the treadmill but this encouragement can lead to bias due to the difficulty of applying prodding evenly to every subject mouse.

a. Procedure

All observations should be made during the dark/active cycle of the mice. Mice do not need to be single-housed prior to this procedure but should be allowed to acclimate for at least 24 h to the procedure room. Prior to experimentation, mice should be trained daily for 3 days at

speeds of 14-20 m/min (speed depends on mouse age and strain). Mice that cannot learn the treadmill task should not be included in experimental studies. Training sessions should last until mouse exhaustion (1-2 hrs). Immunobehavioral stimulation is delivered 24 h after the final training session. Testing is initiated by conducting a treadmill run to exhaustion. Exhaustion is considered as a cease in running that is not motivated by protective end contact. Time to exhaustion is the measured end point. Distance to exhaustion can be calculated from the time run and velocity of the treadmill.

G) CONCLUSION

Behavioral testing is a fundamental element of PNI research and mice provide a powerful tool for exploring the origins and relevance of sickness symptoms. Like with any experimental procedure, uniform agreement on exact technique between scientists has not been achieved. Therefore, the above should be considered an overview of how to measure sickness, depressive/anxietal, cognitive and physical activity behaviors. As important as appropriate procedures are to successful behavioral testing, pre-experiment considerations are likely the greatest determinate to relevance and reproducibility. It is essential that mice be housed in environments devoid of negative stressors and be well-adapted to any change. Variation in the equipment and experimental design is usually irrelevant when compared to unexpected and unpredictable housing conditions. In fact, wet cages, noise and unfamiliar odors are often used as elicitors of adverse biobehaviors. Thus, consistency, concern and care in handling mice affords the best foundation for success. Finally, keen observation is an additional reward, and making sure to note unanticipated or unusual behaviors during testing may lead to innovative discoveries toward the creation of new behavioral tests and immunobehavioral paradigms.

VII SUMMARY

There is plenty of evidence in the literature supporting the role of IR and hypoxia in the initiation of proinflammation. However, several questions still remain. Most of the IR studies performed to date have focused on end points weeks to months after exposure. Additionally, with the exception of the one or two studies that used very low dose IR, most of the doses tested are in the 10+ Gy range. The same is true for hypoxia. Many of the investigations to date have relied on severe hypoxic episodes where oxygen levels were decreased by at least 50% from normoxia. What remains unknown is how do these treatments affect neuroimmunity and/or overall physiology? Might they both be beneficial to the host? There are a small number of reports suggesting that low grade hypoxia or radiation can have beneficial effects. An example with hypoxia showed that moderate normobaric hypoxia (equivilent to 3000 m, or approximately 74% of sea level oxygen) can lead to the beneficial effect of improved running economy (RE; indicated by the oxygen cost while running at submaximal speeds) (176-177). Evidence for beneficial effects of IR come from Trott's review on very low dose IR exposure. IR also has shown beneficial effect for non-malignant conditions such as psoriasis and eczema with fewer short and long term side effects compared to currently used effective treatments (178). Taken together, these findings indicate two things: 1) that treatment with low grade hypoxia or low dose IR results in a physiological effect, and 2) that treatments at very low doses may actually have beneficial health effects.

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CHAPTER TWO[b]

THE BIOBEHAVIORAL AND NEUROIMMUNE IMPACT OF LOW-DOSE IONIZING RADIATION

I ABSTRACT

In the clinical setting, repeated exposures (10-30) to low-doses of ionizing radiation (≤ 200 cGy), as seen in radiotherapy for cancer, causes fatigue. Almost nothing is known, however, about the fatigue inducing effects of a single exposure to environmental low-dose ionizing radiation that might occur during high-altitude commercial air flight, a nuclear reactor accident or a solar particle event (SPE). To investigate the short-term impact of low-dose ionizing radiation on mouse biobehaviors and neuroimmunity, male CD-1 mice were whole body irradiated with 50 cGy or 200 cGy of gamma or proton radiation. Gamma radiation was found to reduce spontaneous locomotor activity by 35% and 36%, respectively, 6 h post irradiation. In contrast, the motivated behavior of social exploration was un-impacted by gamma radiation. Examination of pro-inflammatory cytokine gene transcripts in the brain demonstrated that gamma radiation increased hippocampal TNF-α expression as early as 4 h post-irradiation. This was coupled to subsequent increases in IL-1RA (8 h and 12 h post irradiation) in the cortex and hippocampus and reductions in activity-regulated cytoskeleton-associated protein (Arc) (24 h post irradiation) in the cortex. Finally, restraint stress was a significant modulator of the neuroimmune response to radiation blocking the ability of 200 cGy gamma radiation from impairing locomotor activity and altering the brain-based inflammatory response to irradiation.

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Taken together, these findings indicate that low-dose ionizing radiation rapidly activates the neuroimmune system potentially causing early onset fatigue-like symptoms in mice.

II INTRODUCTION

The impact of ionizing radiation on behavior and neuroimmunity is an emerging field. Currently, the primary focus is on clinically delivered radiation therapy to oncology patients and the consequent adverse biobehavioral impact these critical treatments engender (1-3). Therapeutic radiation involves delivery of relatively high doses (30-80 Gy/14-60 days) administered focally and strategically to limit treatment of uninvolved normal tissues (4). The majority of patients receiving radiotherapy over the past century have been treated with electrons, x-rays (high energy photons) or gamma-rays (high energy photons). The distinction between x- and gamma-rays from a radiotherapy perspective relates to the source of the photons: x-rays originate from outer electrons and gamma rays originate from atomic nuclei. In radiotherapy applications, however, both x- and gamma-rays are photons in the 1-20 MeV energy range. The physical properties of these forms of radiation cause maximal energy deposition (dose) to occur early in the tissue particle track at depths of 0.5-4 cm. Recently, there has been renewed interest in treating patients with heavier charged particles such as protons, which deposit the majority of their dose toward the end of the tissue particle track at depths up to 20-30 cm. This affords more focused delivery of dose to deeply seated neoplasms with less radiation being administered to tissues more distal to the target (5). In addition to the differences in macroscopic dose distribution, photons and protons create disparate microscopic dose distributions due to dissimilar linear energy transfer (LET) coefficients. Importantly, differences

in microdosimetric track structure may cause photons and protons to have qualitatively and quantitatively unequal dose-toxicity profiles (6).

In contrast, environmental radiation exposure is ubiquitous, of very low-dose (approximately 6.2 mGy/year) (7), whole body and comprised of a mix of particle types that include photons, electrons, protons and heavy ions. This environmental dosage, however, can jump significantly at altitudes that commercial aircraft fly (approximately 6.30-6.79 μGy/h (32)), in manned space exploration (approximately 50-100 μGy/day during interplanetary travel and 25-50 μGy/day on planetary surfaces (8)) or during a severe nuclear reactor accident such as occurred at Fukishima Daiichi Nuclear Power Plant in Japan 2011 (ground air as high as 1 Gy) (9-10). Environmental radiation can be markedly compounded during a solar particle event (SPE) with doses reaching 1.4 Gy/h for skin, 0.8 Gy/h for eyes and 8 cGy for bone marrow in data modeling studies from the August 1972 SPE (11). In addition, SPE irradiation is primarily comprised of relatively superficially penetrating protons with energies less than 50 MeV. The energy spectra a specific SPE, however, is highly variable and some SPES have had a greater proportion of deeply penetrating, higher energy protons. With the anticipation of expanded near space/space tourism/travel, nuclear power plant construction and threat of nuclear terrorism, the population at risk for total body radiation exposures in the range of 0.5-2 Gy are likely to increase appreciably.

Total body exposure to ionizing radiation can lead to acute radiation syndrome (ARS) that includes the initial prodromal stage defined by nausea, vomiting and diarrhea (N-V-D stage) (19). An underappreciated component of the prodromal stage is neuroimmune system-mediated sickness symptoms often described as feelings of unease and weakness with an associated lack of motivation and energy (2,12-13). Like other maladies associated with weariness and malaise,

radiation-induced fatigue is a complex interplay of mental, emotional and physical biobehaviors that are often ignored due to concerns over the manifest illness stage and, ultimately, survival. The first radiation-induced behavioral effects involving the dose and type(s) of radiation present in SPEs were delineated in animals (predominantly primates) during the 1970s and 80s. Memory and cognition testing in monkeys irradiated at dose rates of 0.3, 0.8 and 1.8 Gy/min, (total dose of 10.0 Gy) showed that hampered performance occurred in 81% of animals at 1.8 Gy/min but only in 7% of animals at 0.3 Gy/min. Thus, the effective dose for radiation-induced performance deficits was estimated to occur at doses of 3 Gy or less (14). In addition, behavioral test complexity appeared impacted by ionizing radiation with tasks requiring greater physical exertion being affected more (14). As for rodents, conditioned taste aversion could be induced at doses as low as 0.25 Gy (14). Interestingly, 3 Gy of proton radiation caused a decrease in latency to fall in rotarod testing and loss of acoustic startle habituation (15). In sum, almost all studies reporting on the behavioral impact of low-dose radiation (≤10 Gy) examined endpoints of days to weeks post radiation. Therefore, almost nothing is known about the immunobehavioral impact of low-dose ionizing radiation within hours after exposure.

III METHODS

Materials. All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except as noted. RNAlater (AM7021) and RiboPure Blood Kits (AM1928) were purchased from Ambion (Austin, TX). QIAGEN RNeasy Lipid Tissue Mini Kits (Cat No. 74804) were purchased from QIAGEN (Valencia, CA). Reverse transcription kits and primers for qPCR were

purchased from Applied Biosystems (Foster City, CA). Plastic containment cubes (AMAC530C) were purchased from AMAC Plastic Products (Petaluma, CA).

Animals. Animal use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). Male 6-wk old CD-1 mice (n=632) were obtained from Taconic (Hudson, NY). Mice were group housed (4 x cage) in standard shoebox cages (length 28 cm; width 17 cm; height 12.5 cm) and allowed water and standard rodent chow *ad libitum*. Mice were maintained in an environmentally controlled room on a 12 h dark/light cycle (1900 h-0700 h) at a temperature of 72º F (22 º C) and a humidity of 45-55%. Mice tested were 7-8 wks of age. Video recording of animal behavior was performed under red light using a Sony HDR-XR500V Night Shot capable video camera (San Diego CA, USA).

Radiation exposure. Mice were singly housed for 12 h prior to irradiation or sham irradiation. ^{137}Cs irradiated mice were exposed to gamma radiation for no more than 10 min at a dose rate of 44.50 ± 0.1 cGy/min (high dose rate) to doses of 50 cGy or 200 cGy. ^{137}Cs radiation was delivered using a Nordion GammaCell 40-Series 1 Irradiator (Ottawa, Canada). ^{60}Co irradiated mice were exposed to gamma radiation at a dose rate of 0.5 ± 0.01 cGy/min (low-dose rate). ^{60}Co radiation was delivered using an Atomic Energy of Canada Eldorado Model 'G' irradiator (Ottawa, Canada). Proton irradiated mice were exposed to proton radiation at a dose rate of either 50.0 ± 0.1 cGy/min (high-dose rate) or 0.5 ± 0.01 cGy/min (low dose rate). Proton radiation was delivered using the horizontal clinical beam line at the Loma Linda University Medical Center (LLUMC, Loma Linda CA). Protons were tailored to have a similar macroscopic dose distribution as ^{60}Co produced photons, which are considered to be the standard by which other forms of therapeutic radiation are compared (6,16). Sham irradiated mice were treated

identically to irradiated mice except the radiation source remained shielded. Irradiation occurred 3 h prior to the start of the dark cycle (1600 h).

Restraint. Immediately before radiation exposure or sham irradiation exposure, all subject mice were individually placed inside 7.25 cm x 4.10 cm x 4.10 cm well-ventilated containment cubes. These cubes did not provide absolute restraint and mice were able to move minimally. In minimally restrained mice, time of confinement did not exceed 10 min (restraint-10 mice). For mice subjected to more sustained restraint, confinement in the containment cube was 240 min (restraint-240 mice). Therefore, restraint-10 mice were irradiated while simultaneously confined (total confinement/irradiation duration $\leq$ 10 min). Restraint-240 mice were either: 1) irradiated while simultaneously confined (confined/irradiation duration, 10 min) then allowed to remain in the containment cube sans irradiation for an additional 230 min (total confinement, 240 min) or 2) irradiated while simultaneously confined (total confinement/irradiation duration, 240 min). Following any form of restraint, mice were returned to their home cage.

Social exploration. Social exploration was performed as we have previously described (17). Social exploration testing was initiated 10 min after irradiation/restraint exposure. At the time points indicated a 3-4 wk-old novel conspecific juvenile mouse of the same sex (challenge mouse) was introduced into the home cage of the subject mouse. The challenge mouse was confined in a 7.62 x 7.62 x 7.62 cm square metal mesh enclosure. Testing duration was 5 min and a new challenge mouse was used to test each subject mouse at every time point examined. Investigation/exploration was evaluated from the video record and was considered as nose-to-enclosure contact.

Locomotion. Spontaneous locomotor activity was measured as we have previously described (18). Immediately after irradiation/restraint exposure and at the time points indicated mice were video recorded in their home cage for 5 min. Movement was quantified using EthoVision XT 7 (Noldus Information Technology, Leesburg VA). Parameters examined included distance moved (cm) and velocity of movement (cm/s).

qPCR. Following behavioral testing, mice were sacrificed via CO_2 asphyxiation. For blood collection, cardiac puncture was executed using a Becton Dickinsin (BD) 26G x 3/8 inch needle (Franklin Lakes, NJ). Drawn blood was anti-coagulated in EDTA containing Microtainers (BD, Cat No. 365974). After anticoagulation, 0.4 mL of blood was mixed in 1.3 mL of RNAlater. Total RNA was extracted using RiboPure Blood Kits per manufacturer's instructions. For brain collection, brains were harvested and either perfused or not perfused (un-perfused) (as indicated) with ice cold PBS to remove blood as we have done previously (18). Perfusion studies were performed to determine the contribution, if any, of blood based gene transcripts to brain biomarker detection. Where indicated cortex, hippocampus, hypothalamus and cerebellum were separately dissected from perfused brains as we have done previously (18). In all brain isolations, olfactory bulb was not included for study. Total RNA was extracted using a QIAGEN RNeasy Lipid Tissue Mini Kit per the manufacturer's instructions. After all RNA extractions reverse transcription was performed with an Applied Biosystem high-capacity cDNA reverse transcription kit (Cat No. 4368813). As indicated, qPCR utilized the following TaqMan (Applied Biosystems) gene expression primers: IL-1β (Mm99999061_mH), TNF-α (Mm00443258_m1), IL-1RA (Mm01337566_m1), activity-regulated cytoskeleton-associated protein (Arc) (Mm00479619_m1), IL-6 (Mm01210733_m1), IL-1α (Mm_99999060_m1) and IFN-γ (Mm99999071_m1). qPCR was performed on a 7900 HT Fast real-time PCR system (Applied

Biosystems) using TaqMan universal PCR master mix (Cat No. 4318157). To normalize gene expression, a parallel amplification of endogenous glyceraldehydes-3-phosphate dehydrogenase (Mm03302249_g1) was performed. Relative quantitative evaluation of target gene levels was performed by comparing ΔC_t's, where C_t was the threshold concentration.

Statistics. All data are presented as mean ± SEM. Data were analyzed using SAS 9.2 (SAS Institute, Inc., Cary NC). To test for statistical differences, a one-way or two-way ANOVA was used with or without repeated measurements where needed. Tukey's test was used for post-hoc pair-wise multiple comparison procedures. Where needed and indicated, raw data was transformed to attain normal distribution. Also, where indicated, a Freidman's two-way ANOVA for non-parametric analysis was used when nonparametric data was unable to be transformed to a normal distribution. All statistical analysis included testing for time point x dose, restraint x dose or perfusion x dose interactions. Statistical significance was denoted at $p<0.05$.

IV RESULTS

Gamma radiation but not proton radiation reduces mouse locomotor activity. Figure.1A shows that restraint-10 mice exposed to 50 or 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) had, respectively, a 33.8% and 35.1% reduction in spontaneous distance moved (locomotion) 6 h post irradiation compared to sham irradiated mice. Figure.1B shows that mean velocity of movement (velocity) was reduced 6 h post irradiation at both 50 and 200 cGy of gamma radiation by 34.7% and 35.7%, respectively. When 50 or 200 cGy of gamma radiation was delivered at approximately 1/100 the dose rate (0.5 ± 0.01 cGy/min), mouse locomotion/velocity was not impacted at 0, 2, 4, 6, 8 or 24 h after irradiation (data not shown).

Similarly, when 50 or 200 cGy of proton radiation was used (dose rate of either 0.5 ± 0.01 cGy/min or 50.0 ± 0.1 cGy/min), mouse locomotion/velocity was not impacted at 0, 2, 4, 6, 8 or 24 h after irradiation (data not shown). Social exploration was not affected by either 50 or 200 cGy of gamma or proton radiation (regardless of dose rate) at 0, 2, 4, 6, 8 and 24 post irradiation (Table S1).

Gamma radiation up-regulates gene transcripts for TNF-α and Arc in whole brains 6 h post irradiation. Figure.2A shows that unperfused and perfused brains from restraint-10 mice exposed to 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) had a 3.2-fold and 2.1-fold increase in TNF-α gene transcripts, respectively, 6 h post irradiation. Whole brain gene transcript expressions for IL-1α, IL-1β, IL-1RA, IL-6 and IFN-γ were not impacted by gamma radiation at this time point. Figure 2B demonstrates that in perfused brains restraint-10 mice exposed to 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) had a 0.37-fold decrease in Arc 6 h post irradiation.

Gamma radiation up-regulates gene transcripts for IL-1β and IL-1RA in blood 8 h post irradiation. Figure.3A&B show that blood from restraint-10 mice exposed to 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) had a 3.3-fold and 3.3-fold increase in IL-1β and IL-1RA gene transcripts, respectively, 8 h post irradiation. Blood gene transcript expressions for IL-1α, TNF-α, IL-6, and IFN-γ were not impacted by gamma radiation at this time point or at 4 h post gamma irradiation. IL-1α was increased 2.1-fold at 6 h by 200 cGy of gamma radiation (data not shown), however, IL-1β, IL-1RA, TNF-α, IL-6, and IFN-γ were not. Figure.3C&D demonstrate that up-regulation of IL-1β and IL-1RA gene transcripts in whole brain at 8 h post gamma irradiation are due to blood in the brain.

Restraint inhibits the impact of 200 cGy gamma radiation on locomotor activity. Fig.4A demonstrates that in restraint-240 mice 200 cGy of radiation (44.5 ± 0.1 cGy/min) increased locomotion 4 h post irradiation by 20.3% and 25.4%, respectively, compared to sham irradiated restraint-240 mice and restraint-240 mice exposed to 50 cGy of gamma radiation. Likewise, 4 h post irradiation velocity was increased in restraint-240 mice exposed to 200 cGy of gamma radiation by 25.7% and 45.7%, respectively, compared to sham irradiated restraint-240 mice and restraint-240 mice exposed to 50 cGy of gamma radiation. At 6 h post irradiation mice exposed to 50 cGy had a 29.2% and 35.3% decrease in locomotion compared to sham and 200 cGy gamma irradiated mice and a 29.6% and 35.6% reduction in velocity, respectively. When restraint-240 mice were compared to restraint-10 mice, restraint-240 mice exposed to 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) moved 39.5%, 83.5% and 59.1% farther at 4, 6, and 8 h post irradiation, respectively (Table S2). Non-irradiated restraint-240 mice and restraint-10 mice moved similarly except at the 10 h time point (Table S2).

Restraint of gamma irradiated mice impacts TNF-α, IL-1RA and Arc gene expression differentially in cerebral hippocampus, hypothalamus, cortex, and cerebellum at 4, 8, 12 and 24 h post irradiation. Table 1 shows that when restraint-10 mice and restraint-240 mice were exposed to either 50 or 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) differential gene transcript expression of TNF-α, IL-1RA and Arc occurred that was dependent on brain region, restraint duration and dose of gamma radiation.

V DISCUSSION

Near continuous exposure to environmental ionizing radiation of a very low-dose rate is omnipresent. With certain occupations and in certain circumstances, dose rate can increase such that a modest dose of radiation (200 cGy) is received in a relatively short period of time (>8 hr). In humans, these exposures are increasing in frequency (nuclear accidents) and becoming better recognized (SPEs). When ionizing radiation doses are significant in duration or energy to cause ARS, prodromal stage symptoms of nausea, vomiting and diarrhea (NVD) (3,19) occur manifesting within hours (19). Fatigue is either underappreciated or unreported because prodromal stage research has focused on NVD as this symptom triad is perceived as predicative of severe organ damage and demise (19). In humans, radiation-induced NVD typically requires a minimum dose of 70 cGy, although mild symptoms may be observed at doses of 30 cGy (20). Radiation-induced fatigue has been best studied in relationship to radiation therapy where loss of energy and malaise is a common side effect (21). Serious cancer treatment-associated fatigue, however, usually manifests gradually (22) compounding with delivery of repeated fractionated doses of 200 cGy that over the course of therapy (up to 8 wks), can deliver up to 80 Gy. In general, CNS function is not felt to be impacted at single radiation doses of ≤ 200 cGy (23) and if fatigue is documented in a single 100-200 cGy exposure it is usually tallied during the illness phase which for a dose ≤ 200 cGy would occur nearly a month post exposure (23). Overall, a single isolated exposure of less than ≤ 200 cGy is deemed recoverable without supportive care (23) which is underscored by the establishment of 5 cSv (equivalent to 5 cGy of gamma radiation) as the annual occupational radiation exposure limit by the Nuclear Regulatory Commission (NRC) (24).

As delineated above, little work has been performed examining the early impact (<24 h) of low-dose (≤ 2 Gy) ionizing radiation on the neuroimmune system. Fig.1 demonstrates that a single dose of gamma radiation as low as 50 cGy reduces mouse locomotor activity 6 h after exposure. When social exploration was examined at radiation doses of 50 cGy and 200 cGy neither dose impaired mouse exploratory behavior (Table S1). These findings indicate that low-dose ionizing gamma radiation appears to perturb unmotivated behaviors to a greater extent than motivated behaviors. Such results are similar to findings we have observed in high-fat diet (HFD) fed mice where the HFD state causes a decrease in spontaneous locomotion (18) that is not reflected by a loss of social exploration (25). Customarily, strong activators of the neuroimmune system like lipopolysaccharide (LPS) induce both locomotor retardation and social withdrawal (26). As we have shown, HFD-feeding appears to be a very weak stimulator of CNS inflammation (18). Consequently, from a biobehavioral standpoint low-dose radiation is at best a very weak immediate activator of neuroimmunity.

To demonstrate that the irradiation given could activate the neuroimmune system, pro-inflammatory cytokine gene transcripts were measured in whole brains from mice administered both 50 cGy and 200 cGy of gamma radiation. Fig. 2 shows that TNF-α is increased 6 h post irradiation in mice receiving 200 cGy. When un-perfused brains were compared to perfused brains, 50 cGy increased TNF-α transcripts in un-perfused brains. To determine if this boost in transcripts was due to an increase in blood TNF-α transcripts, whole blood TNF-α transcripts were examined and found to be unaffected by gamma radiation. Blood, however, did show significant transcript up-regulation of the inflammatory bioactives IL-1β and IL-1RA 8 h post 200 cGy gamma irradiation (Fig.3) and these blood transcripts were present in the brain (Fig.3).

Taken together these findings indicate that low-dose gamma radiation activates the neuroimmune system relatively rapidly. How radiation triggers this response is not clear.

Previous work with high-dose radiation in mice (15 Gy) has demonstrated that irradiation of the body without irradiating the head induces up-regulation of proinflammatory cytokine transcripts in the brain (12). This work was designed to support the concept that a stimulated peripheral innate immune system could communicate with the brain as is seen with peripheral LPS administration (26). Although not designed to test this question, our work is supportive in that blood present in the brain contains cells with increased IL-1β gene transcripts. Further support for this concept was seen in the proton radiation experiments because proton irradiation at both 50 cGy and 200 cGy did not disturb either locomotion or social exploration (data not shown, Table S1, respectively). Proton radiation is of higher energy than gamma radiation (27) and contributes more significantly to SPEs (6,27). Given this higher energy, we expected proton radiation to impact behavior more than gamma radiation. Proton radiation, however, has a different linear energy transfer profile than gamma radiation. The Bragg peak for gamma radiation is likely more toward the skin surface as opposed to proton radiation where the Bragg peak is likely skewed to the interior of the animal. This difference in ionization location further supports the potential importance of peripheral immune activation to radiation-induced neuroimmune activation. Interestingly, blood also showed an up-regulation of IL-1RA transcripts indicating (in conjunction with the IL-1β transcript data noted above) that the IL-1 arm of the innate immune system is an early pathway activated by radiation. Organ systems with high radiation sensitivity include the hematopoietic system where radiation increases mitochondrial-dependent reactive oxygen species (ROS) generation (28). Importantly, ROSs have been shown recently to stimulate the NALP3 inflammasome (one of three inflammasomes responsible for

activation of caspase-1) which is required for the conversion of pro-IL-1β into mature secretable IL-1β (29). Curiously, little work has been performed examining radiation and inflammasome activation. Given the reduction of locomotion observed after 50 cGy of radiation and the lack of pro-inflammatory transcript changes, radiation-dependent triggering of the inflammasome with production of mature IL-1β, as seen with the NALP3 inflammasome and uric acid (31), seems likely. TNF-α gene transcript up-regulation may be secondary to the IL-1β signal if that signal is significantly robust.

How pro-inflammatory cytokines induce fatigue is still imprecise. Although CNS IL-1 (32) and TNF-α are implicated (33-34), the mechanistic connection remains elusive. Some believe the indoleamine-2,3-dioxygenase (IDO) pathway may be important (36) because altered serotonergic (5-hydroxytryptamine, (5-HT)) neurotransmission is identified in patients with chronic fatigue syndrome (36). In addition, pro-inflammatory cytokines, especially TNF-α, provoke the brain-based IDO pathway to convert the serotonin precursor tryptophan to kynurenine which affords production of potential neurotoxic kynurenine metabolites (37-38). With radiation-induced fatigue, however, the IDO pathway seems an unlikely player because blockade of 5-HT is a key first line defense for the inhibition of radiation-induced nausea and vomiting in radiotherapy patients (39) and there is no clear evidence that inhibition of these prodromal ARS symptoms with 5-HT antagonists ameliorates subsequent radiation-induced fatigue.

Low dose-rate gamma radiation was also examined and found not to perturb locomotor or social exploratory behavior (data not shown, Table S1, respectively). The most likely explanations for these observations were that low-dose rate radiation (50 cGy and 200 cGy of radiation delivered over 240 min as opposed to 10 min as in Fig.1 (high-dose rate)) did not

impart significant energy to activate the neuroimmune system or that the mice in the low-dose rate experiments were restrained for 240 min as opposed to 10 min. To investigate these possibilities, more prolonged restraint experiments were performed in which mice were delivered 50 cGy and 200 cGy (both at high-dose rate) during the first 10 min of restraint then left restrained for an additional 230 min. Fig.4 reveals that restraint-240 ameliorated the effect of 200 cGy on locomotion but not that of 50 cGy. Unexpectedly, restraint-240 produced hyper-mobility in mice irradiated with 200 cGy suggesting that restraint-240 may sensitize mice to biobehavioral stimuli like it does for cutaneous hypersensitivity (40). Importantly, restraint-240 had no impact on locomotor activity of sham irradiated mice (data not shown) which is consistent with the majority of work examining restraint (41). Taken together these findings indicate that early radiation-induced alterations in biobehaviors requires a threshold dose rate that can be potentially modulated by the stress response. Furthermore, since the bulk of the time restrained was spent after irradiation these results point to a radiation priming-like interaction which occurs at 200 cGy but not at 50 cGy because the 50 cGy irradiated restraint-240 mice behaved like restraint-10 50 cGy irradiated mice (Fig.1).

In general, the overall impact of restraint tends towards immune suppression (41). Table 1 shows that immediately after restraint-240 (4 hr post-irradiation) sham irradiated restraint-240 mice had a decrease in hippocampal, hypothalamic, cortical and cerebellar TNF-α transcripts compared to sham irradiated restraint-10 mice. 200 cGy irradiation prevented this down-regulation while 50 cGy had little effect. As time post-irradiation progressed, TNF-α transcripts increased showing a movement toward resolution by 24 hr post irradiation. Unlike whole brain at 8 h post irradiation (Fig 3D), brain regions showed region-specific increases in IL-1RA gene transcripts. At 8 h post-irradiation, hippocampal IL-1RA gene expression was increased

especially in the cortex (Table 1). Peak IL-1RA expression occurred near 12 hrs post-irradiation with resolution before 24 hrs. IL-1RA expression patterns somewhat mimicked that of TNF-α but were expressed in the brain later and resolved quicker. This pattern fits with a NALP3 inflammasome driven biobehavioral process (17).

Finally, activity-regulated cytoskeletal-associated protein (Arc) was up-regulated by restraint-240 in the hippocampus as previously reported (42). Arc is an immediate early gene induced in hippocampal and parietal neurons following behavioral experiences best tied to maintenance of long-term potentiation and spatial memory consolidation (43). Inflammation and proinflammatory cytokines, including TNF-α, are shown to reduce Arc gene transcript expression (44). Unexpectedly, restraint-240 led to decreased Arc in the cerebellum. What function Arc has in the cerebellum is not clear but it may play a role in cerebellar associative learning as evoked in such responses as eyeblink conditioning (45). 200 cGy gamma irradiation led to a decrease in whole brain Arc gene transcripts at 6 hr. Brain region analysis (Table 1) showed that this effect was transient in that it was not evident at 4 hr or 8 hr post irradiation. At 24 hr post irradiation (restraint-10 200 cGy), Arc transcripts were reduced in the cortex which may fit with post-radiation cognitive deficits that can manifest after whole brain irradiation, although acute radiation encephalopathy is rare at doses under 300 cGy (46). Taken together these findings indicate that low-dose gamma radiation affects the cortex and hippocampus of mice with changes that can last at least 24 hr post-irradiation. Up-regulated TNF-α appears linked to down-regulated Arc. Since manned space and commercial air travel carry a significant risk of SPE exposure in conjunction with physical and psychological stress (extensively studied in astronauts (47) and more recently recognized in airline passengers (economy class syndrome (48-49))), restraint stress may modulate early radiation-induced fatigue due to brain-based

immunosuppression (50). However, this restraint-240-dependent reduction in TNF-α gene expression is short-lived and cortical TNF-α is higher in irradiated restraint-240 animals at 12 hr and 24 hr post irradiation when compared to restraint-10 animals. More work is required to understand if and/or how early neuroimmune activation contributes to radiation-induced fatigue-like responses, especially when conjoined to the stress response.

VI FIGURES

A) FIGURE 2.1

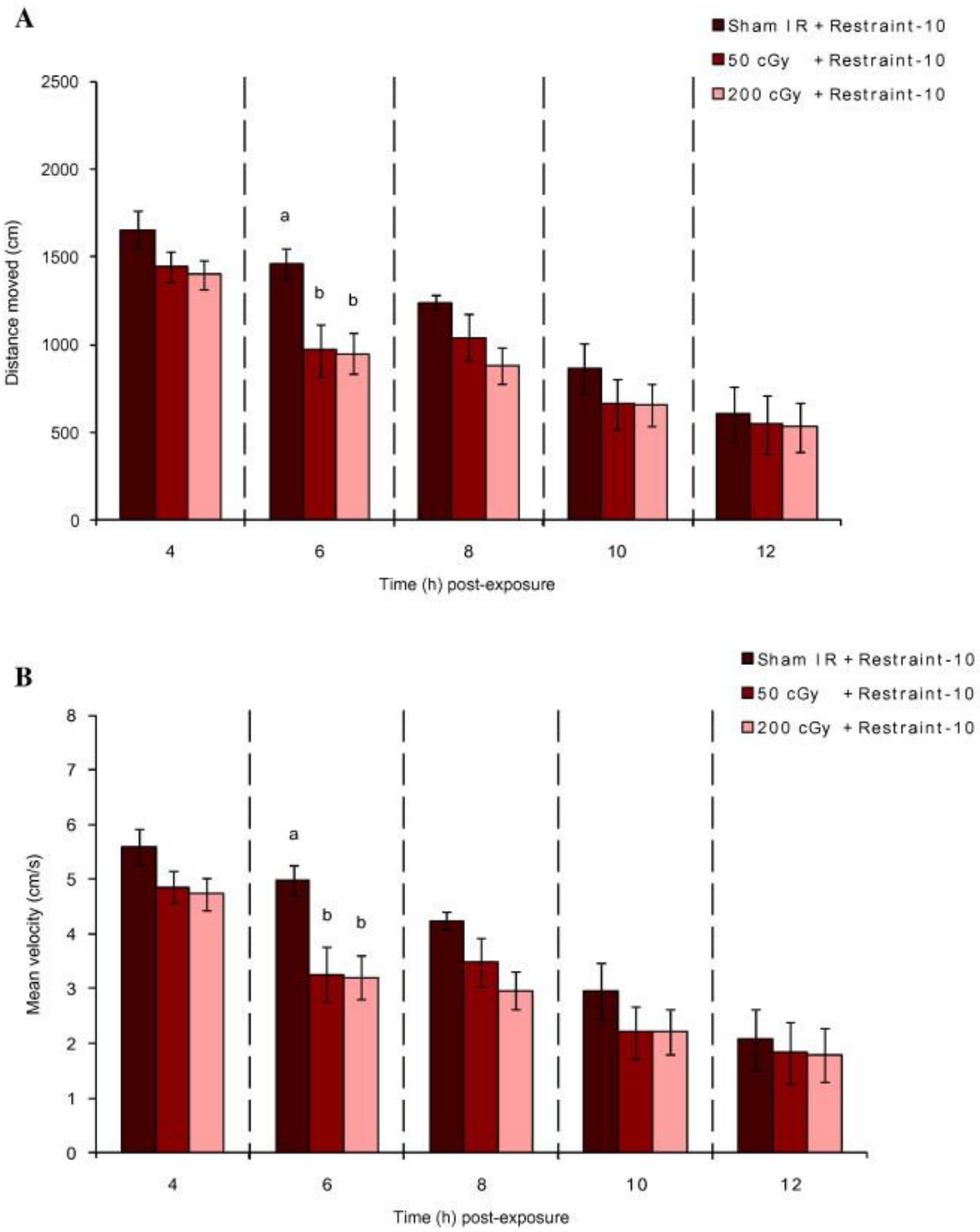

Figure 2.1. Gamma radiation but not proton radiation reduces mouse locomotor activity. Restraint-10 mice were exposed to 50 or 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) as indicated. Spontaneous locomotor activity and velocity of movement were measured at the time points indicated post irradiation. Results are expressed as means ± s.e.m.; n = 8. (*A*) Distance moved (cm): main effects of dose ($P < 0.001$) and time point ($P < 0.001$); 6 h time point: $P < 0.05$, sham IR *v*. 50 cGy (1463.8 ± 83.7 *v*. 968.4 ± 147.6) and sham IR *v*. 200 cGy (1463.8 ± 83.7 *v*. 950.5 ± 117.2). (*B*) Velocity of movement (cm/s): main effects of dose ($P < 0.001$) and time point ($P < 0.001$); 6 h time point: $P < 0.05$, sham IR *v*. 50 cGy (5.0 ± 0.3 *v*. 3.3 ± 0.5) and sham IR *v*. 200 cGy (5.0 ± 0.3 *v*. 3.2 ± 0.4). Bars without a common superscript are different ($P < 0.05$).

B) FIGURE 2.2

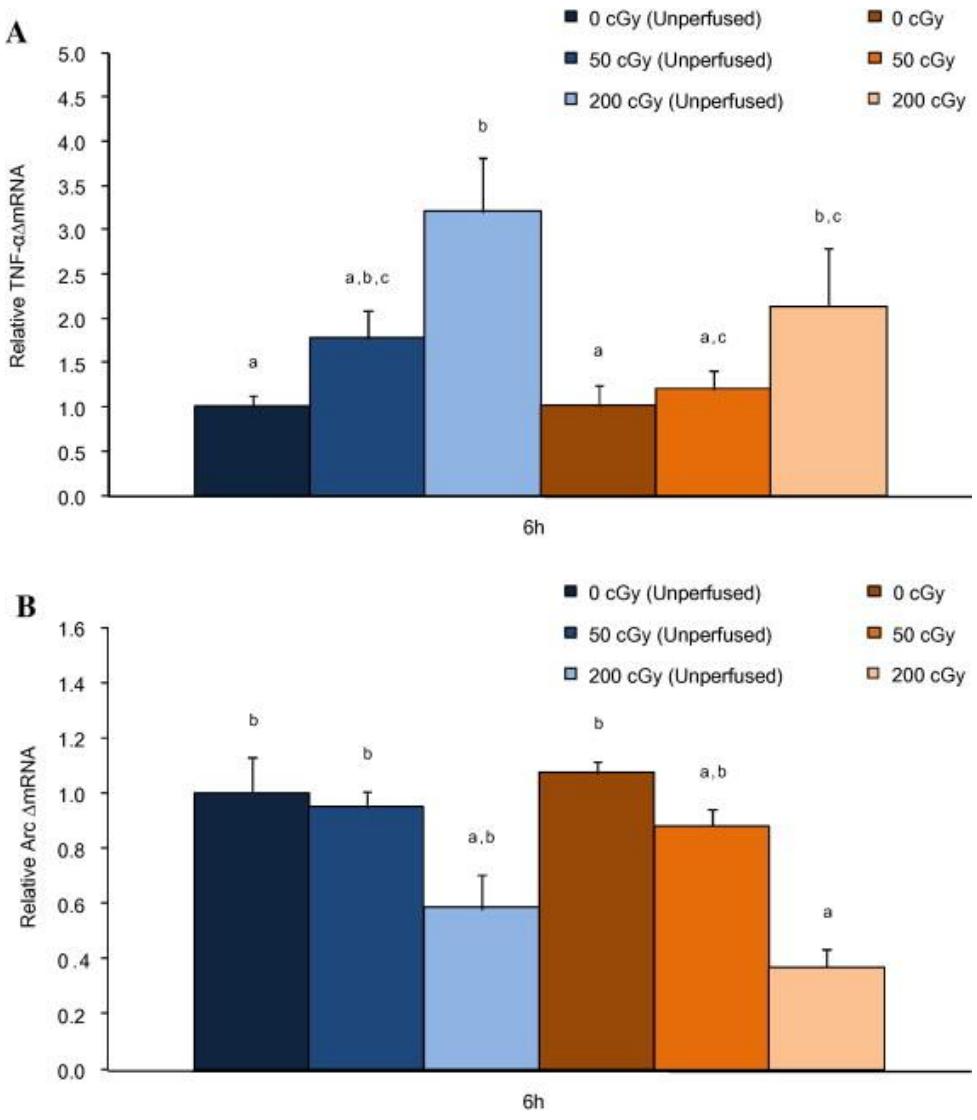
A
0 cGy (Unperfused)
50 cGy (Unperfused)
200 cGy (Unperfused)
0 cGy
50 cGy
200 cGy
Relative TNF-α ΔmRNA
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b,c
b
a
a,c
b,c
6h
B
0 cGy (Unperfused)
50 cGy (Unperfused)
200 cGy (Unperfused)
0 cGy
50 cGy
200 cGy
Relative Arc ΔmRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
b
a,b
b
a,b
a
6h

Figure 2.2. Gamma radiation up-regulates gene transcripts for TNF-α and Arc in whole brain 6 h post irradiation. Restraint-10 mice were exposed to 50 or 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) as indicated. qPCR was used to quantify mRNAs from un-perfused and perfused whole brains as indicated 6 h post irradiation. Results are expressed as relative fold change in mRNA expression (ΔmRNA), means ± s.e.m.; n = 4-6. (*A*) TNF-α: main effect of dose ($P < 0.001$) and perfusion ($P = 0.042$); $P < 0.05$, 200 cGy (unperfused) *v*. sham IR (unperfused), sham IR, 50 cGy (3.205 ± 0.252 *v*. 1.000 ± 0.166, 1.017 ± 0.286, 1.201 ± 0.241, respectively); $P < 0.05$, 200 cGy *v*. sham IR (unperfused), sham IR (2.139 ± 0.387 *v*. 1.000 ± 0.166, 1.017 ± 0.286, respectively). (*B*) Arc: main effect of dose ($P = 0.003$); $P < 0.05$, 200 cGy *v*. sham IR (unperfused), 50 cGy (unperfused), sham IR (0.370 ± 0.076 *v*. 1.000 ± 0.239, 0.951 ± 0.211, 1.074 ± 0.107, respectively).

C) FIGURE 2.3

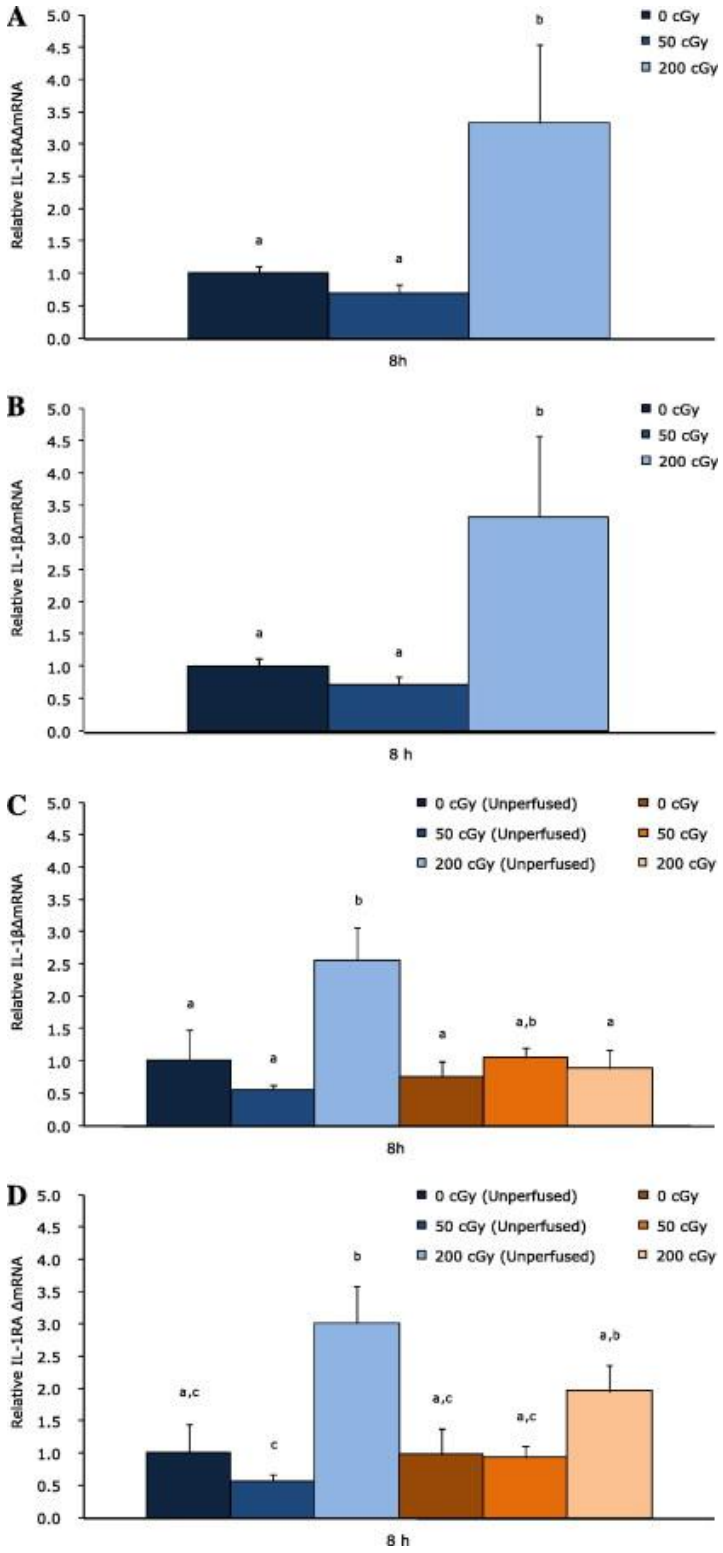
A
Relative IL-1RAΔmRNA
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
8h
0 cGy
50 cGy
200 cGy
B
Relative IL-1βΔmRNA
a
a
b
8 h
0 cGy
50 cGy
200 cGy
C
Relative IL-1βΔmRNA
a
a
b
a
a,b
a
8h
0 cGy (Unperfused)
50 cGy (Unperfused)
200 cGy (Unperfused)
0 cGy
50 cGy
200 cGy
D
Relative IL-1RA ΔmRNA
a,c
c
b
a,c
a,c
a,b
8 h
0 cGy (Unperfused)
50 cGy (Unperfused)
200 cGy (Unperfused)
0 cGy
50 cGy
200 cGy

Figure 2.3. Gamma radiation up-regulates gene transcripts for IL-1β and IL-1RA in blood 8 h post irradiation. Restraint-10 mice were exposed to 50 or 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) as indicated. qPCR was used to quantify mRNAs from blood and un-perfused and perfused whole brains as indicated 8 h post irradiation. Results are expressed as relative fold change in mRNA expression (ΔmRNA), means ± s.e.m.; n = 4-6. (*A*) IL-1β: main effect of dose ($P < 0.001$); $P < 0.05$, 200 cGy *v*. sham IR, 50 cGy (3.312 ± 0.468 *v*. 1.000 ± 0.182, 0.711 ± 0.254, respectively). (*B*) 8 h IL-1RA: main effect of dose ($P < 0.001$); $P < 0.05$, 200 cGy *v*. sham IR, 50 cGy (3.335 ± 0.446 *v*. 1.000 ± 0.146, 0.690 ± 0.273, respectively). (*C*) IL-1β: main effect of dose (P = 0.006), dose-perfusion interaction (P = 0.002); P < 0.05, 200 cGy (unperfused) *v*. sham IR (unperfused), 50 cGy (unperfused), sham IR , 50 cGy (2.555 ± 0.267 *v*. 1.000 ± 0.570, 0.557 ± 0.182, 0.755 ± 0.407, 1.052 ± 0.189, respectively). (*D*) IL-1RA: main effect of dose (P < 0.001); P < 0.05, 200 cGy (unperfused) *v*. sham IR (unperfused), 50 cGy (unperfused), sham IR, 50 cGy (3.013 ± 0.255 *v*. 1.000 ± 0.531, 0.570 ± 0.244, 0.982 ± 0.488, 0.935 ± 0.258, respectively); P = 0.009, 200 cGy *v*. 50 cGy (unperfused) (1.961 ± 0.267 *v*. 0.570 ± 0.244). Bars without a common superscript are different (P < 0.05).

D) FIGURE 2.4

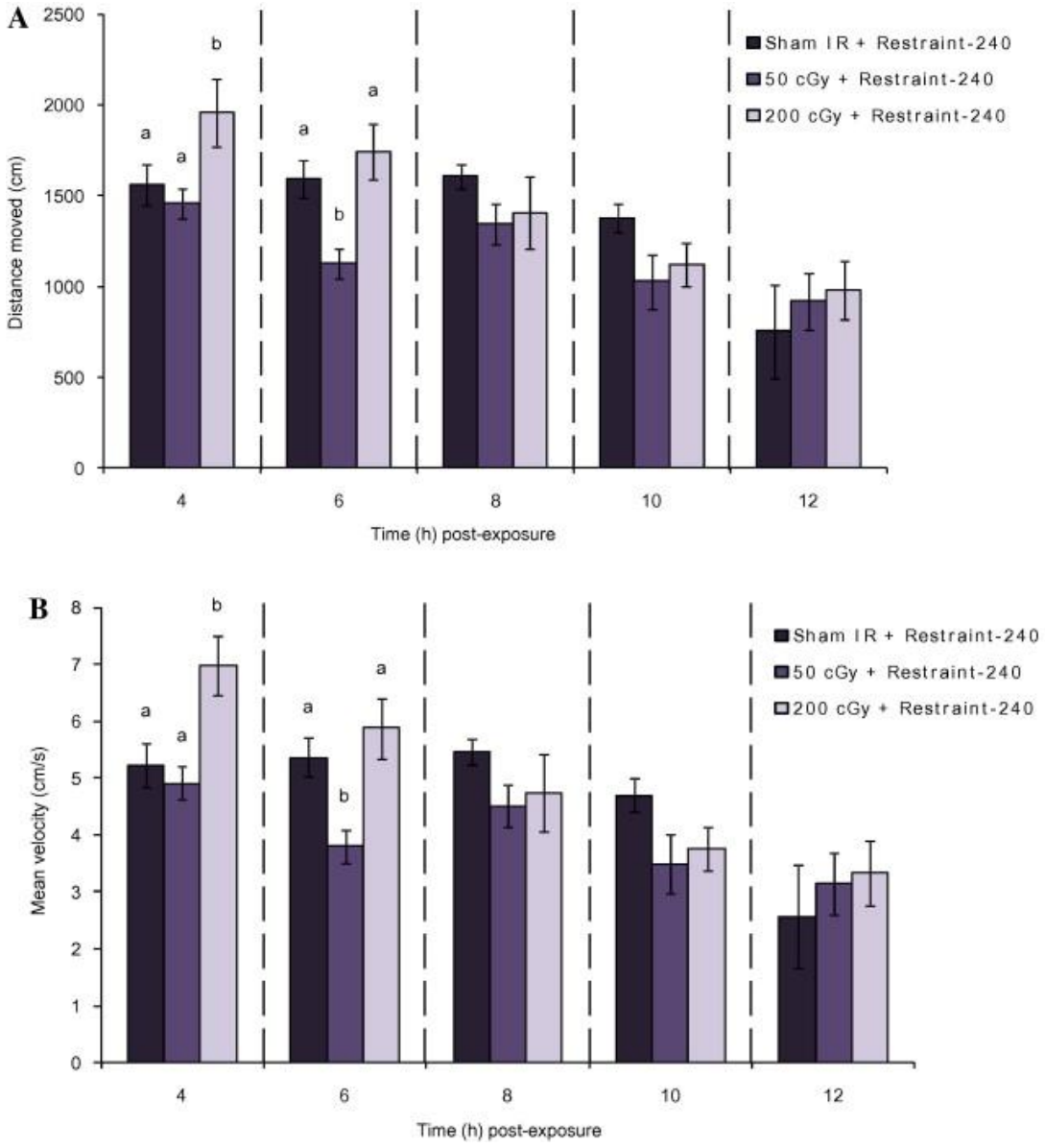
A
Distance moved (cm)
2500
2000
1500
1000
500
0
a
a
b
a
b
a
4
6
8
10
12
Time (h) post-exposure
Sham IR + Restraint-240
50 cGy + Restraint-240
200 cGy + Restraint-240
B
Mean velocity (cm/s)
8
7
6
5
4
3
2
1
0
a
a
b
a
b
a
4
6
8
10
12
Time (h) post-exposure
Sham IR + Restraint-240
50 cGy + Restraint-240
200 cGy + Restraint-240

Figure 2.4. Restraint-240 inhibits the impact of 200 cGy gamma radiation on locomotor activity. Restraint-240 mice were exposed to 50 or 200 cGy of gamma radiation (44.5 ± 0.1 cGy/min) as indicated. Spontaneous locomotor activity and velocity of movement were measured at the time points indicated post irradiation. Results are expressed as means ± s.e.m.; n = 8-16**.** (*A*) Distance moved (cm): main effects of dose ($P = 0.004$) and time point ($P < 0.001$), dose-time point interaction ($P = 0.036$); 4 h time point: $P \leq 0.05$, sham IR + restraint-240 *v*. 200 cGy + restraint-240 (1559.1 ± 111.5 *v*. 1955.8 ± 188.1), 50 cGy + restraint-240 *v*. 200 cGy + restraint-240 (1457.3 ± 85.6 *v*. 1955.8 ± 188.1); 6 h time point: $P < 0.05$, sham IR + restraint-240 *v*. 50 cGy + restraint-240 (1592.9 ± 101.8 *v*. 1127.7 ± 82.0), 50 cGy + restraint-240 *v*. 200 cGy + restraint-240 (1127.7 ± 82.0 *v*. 1743.9 ± 151.2). (*B*) Velocity of movement (cm/s): main effects of dose ($P =0.003$) and time point ($P < 0.001$), dose-time point interaction ($P = 0.013$); 4h time point: $P < 0.05$, sham IR + restraint-240 *v*. 200 cGy + restraint-240 (5.2 ± 0.4 *v*. 7.0 ± 0.5), 50 cGy + restraint-240 *v*. 200 cGy + restraint-240 (4.9 ± 0.3 *v*. 7.0 ± 0.5); 6 h time point: $P < 0.05$, sham IR + restraint-240 *v*. 50 cGy + restraint-240 (5.4 ± 0.4 *v*. 3.8 ± 0.3), 50 cGy + restraint-240 *v*. 200 cGy + restraint-240 (3.8 ± 0.3 *v*. 5.9 ±0.5). Bars without a common superscript are different ($P < 0.05$).

Table 2.1

Impact of high-dose rate gamma irradiation on TNF-α, IL-1RA and Arc gene transcripts in cerebral hippocampus, hypothalamus, cortex, and cerebellum at 4, 8, 12 and 24 h post irradiation.

			Restraint-10			Restraint-240		
Brain region	**Time post-irradiation (h)**	**Gene**	**Sham IR**	**50 cGy**	**200 cGy**	**Sham IR**	**50 cGy**	**200 cGy**
Hippocampus	**4**	**TNF-α*#**	**1.000 (0.253)^{a,c}**	**1.144 (0.133)^{a,c}**	**1.535 (0.211)^{a}**	**0.394 (0.261)^{b,c}**	**0.599 (0.075)^{c}**	**1.326 (0.450)^{a}**
Hippocampus	**4**	**Arc#**	**1.000 (0.132)^{a}**	**0.886 (0.098)^{a}**	**0.974 (0.147)^{a}**	**1.730 (0.172)^{b}**	**1.303 (0.184)^{a,b}**	**1.326 (0.177)^{a,b}**
Hypothalamus	**4**	**TNF-α*#**	**1.000 (0.320)^{a}**	**0.985 (0.260)^{a,b}**	**1.019 (0.180)^{a}**	**1.235 (0.355)^{b,c}**	**1.248 (0.127)^{c}**	**1.260 (0.339)^{a}**
Hypothalamus	**4**	**IL-1RA#**	**1.000 (0.437)^{a,b}**	**0.668 (0.199)^{a,b}**	**1.313 (0.137)^{a}**	**0.630 (0.381)^{a,b}**	**0.560 (0.194)^{b}**	**0.683 (0.275)^{a,b}**
Cortex	**4**	**TNF-α*#†**	**1.000 (0.237)^{a}**	**1.076 (0.137)^{a}**	**1.226 (0.184)^{a}**	**0.420 (0.220)^{b}**	**0.516 (0.110)^{b}**	**1.096 (0.356)^{a}**
Cortex	**4**	**Arc*#**	**1.000 (0.154)^{a,b,c}**	**0.573 (0.157)^{a}**	**0.667 (0.127)^{a,c}**	**1.732 (0.375)^{b}**	**1.426 (0.227)^{b}**	**1.199 (0.272)^{b,c}**
Cerebellum	**4**	**TNF-α*#**	**1.000 (0.121)^{a}**	**0.963 (0.131)^{a}**	**1.164 (0.198)^{a}**	**0.522 (0.146)^{b}**	**0.465 (0.233)^{b}**	**0.822 (0.352)^{a,b}**
Cerebellum	**4**	**Arc#¶**	**1.000 (0.062)^{a}**	**0.923 (0.042)^{a}**	**0.895 (0.092)^{a}**	**0.532 (0.083)^{c}**	**0.573 (0.130)^{b,c}**	**0.786 (0.147)^{a,b}**
Hippocampus	**8**	**TNF-α***	**1.000 (0.127)^{a}**	**1.338 (0.094)^{a}**	**2.093 (0.136)^{b}**	**1.002 (0.146)^{a}**	**1.200 (0.131)^{a}**	**1.965 (0.138)^{b}**
Hippocampus	**8**	**IL-1RA***	**1.000 (0.491)^{a,c}**	**0.909 (0.204)^{a}**	**1.880 (0.203)^{b,c}**	**1.537 (0.249)^{a,b,c}**	**1.008 (0.203)^{a,c}**	**2.280 (0.103)^{b}**
Hippocampus	**8**	**Arc#**	**1.000 (0.127)^{a,b}**	**0.972 (0.108)^{a,b}**	**1.124 (0.159)^{a}**	**0.904 (0.141)^{a,b}**	**0.782 (0.094)^{b}**	**0.869 (0.106)^{a,b}**
Hypothalamus	**8**	**Arc#†**	**1.000 (0.088)^{a}**	**1.066 (0.087)^{a}**	**0.770 (0.194)^{a,b}**	**0.610 (0.111)^{b,c}**	**0.473 (0.205)^{c}**	**0.674 (0.118)^{b,c}**
Cortex	**8**	**TNF-α***	**1.000 (0.224)^{a,b}**	**1.092 (0.094)^{a,b}**	**1.645 (0.264)^{a}**	**0.732 (0.140)^{b}**	**1.395 (0.336)^{a}**	**1.454 (0.212)^{a}**
Cortex	**8**	**IL-1RA*¶**	**1.000 (0.159)^{a}**	**1.109 (0.067)^{a}**	**3.043 (0.196)^{b}**	**1.199 (0.183)^{a}**	**1.188 (0.157)^{a,b}**	**2.630 (0.085)^{b}**
Hippocampus	**12**	**TNF-α*†**	**1.000 (0.176)^{a}**	**1.497 (0.146)^{b,c}**	**1.890 (0.136)^{c}**	**1.123 (0.123)^{a,b}**	**1.173 (0.111)^{a,b}**	**2.409 (0.152)^{c,d}**
Hippocampus	**12**	**IL-1RA***	**1.000 (0.261)^{a}**	**1.873 (0.241)^{b,}**	**2.391 (0.210)^{b}**	**1.731 (0.167)^{a,b}**	**1.692 (0.275)^{a,b}**	**2.991 (0.123)^{b}**
Cortex	**12**	**TNF-α*#**	**1.000 (0.191)^{a}**	**1.131 (0.172)^{a,b}**	**1.732 (0.149)^{b}**	**1.131 (0.202)^{a,b}**	**1.154 (0.165)^{a,b}**	**2.899 (0.205)^{c}**
Cortex	**12**	**IL-1RA*†¶**	**1.000 (0.076)^{a}**	**1.157 (0.055)^{a}**	**2.454 (0.067)^{b}**	**1.374 (0.113)^{a}**	**1.228 (0.117)^{a}**	**3.499 (0.113)^{b}**
Hippocampus	**24**	**TNF-α***	**1.000 (0.110)^{a}**	**1.109 (0.071)^{a,b}**	**1.529 (0.158)^{b,c}**	**1.050 (0.070)^{a,b}**	**1.166 (0.263)^{a,b}**	**1.807 (0.147)^{c}**
Hippocampus	**24**	**IL-1RA***	**1.000 (0.218)^{a,b}**	**0.804 (0.306)^{a}**	**1.133 (0.159)^{a,b}**	**0.894 (0.175)^{a,b}**	**1.049 (0.337)^{a,b}**	**1.524 (0.097)^{b}**
Cortex	**24**	**TNF-α***	**1.000 (0.227)^{a}**	**1.108 (0.100)^{a,c}**	**1.803 (0.154)^{b,c}**	**1.073 (0.146)^{a}**	**1.139 (0.254)^{a,c}**	**2.332 (0.184)^{b}**
Cortex	**24**	**IL-1RA*†**	**1.000 (0.215)^{a,b}**	**0.662 (0.169)^{a}**	**1.054 (0.141)^{a,b}**	**0.697 (0.164)^{a}**	**0.855 (0.304)^{a}**	**1.580 (0.123)^{b}**
Cortex	**24**	**Arc*†**	**1.000 (0.191)^{a}**	**0.648 (0.123)^{a,b,c}**	**0.431 (0.060)^{c}**	**0.564 (0.126)^{b,c}**	**0.678 (0.170)^{a,b}**	**0.747 (0.258)^{a,b}**

Results are expressed as relative fold change in mRNA expression (ΔmRNA), means (s.e.m.); $n = 6$. *$P < 0.05$, significant main effect of dose. #$P < 0.05$, significant main effect of restraint-240. †$P < 0.05$, significant dose-restraint-240 interaction. Results within individual rows without a common superscript letter are different ($P < 0.05$). "¶" indicates data was transformed using ((Original value)^{2})$^{1/4}$).

Table 2.S1

Impact of radiation on social exploration

Time post-irradiation	Radiation dose	Gamma		Proton	
		Low-dose rate^{+}*	High-dose rate*	Low-dose rate*	High-dose rate^{+}*
0 h	0 cGy	39.4 (7.8)^{a,c}	40.0 (8.9)^{a}	22.0 (4.8)^{a}	37.6 (7.5)^{a,b}
	50 cGy	16.7 (3.7)^{a}	39.1 (6.8)^{a}	25.2 (6.1)^{a,c}	29.0 (6.4)^{a}
	200 cGy	21.9 (4.4)^{a}	52.4 (8.5)^{a,b}	17.9 (5.0)^{a}	35.1 (7.6)^{a,b}
2 h	0 cGy	71.0 (11.6)^{c}	56.0 (9.4)^{a,b,c}	44.8 (8.7)^{a,b,c}	61.4 (12.8)^{a,b}
	50 cGy	55.5 (11.7)^{a,b,c}	64.6 (7.9)^{a,b,c}	45.1 (11.6)^{a,b,c}	43.1 (11.6)^{a,b}
	200 cGy	63.2 (10.7)^{a,b,c}	55.2 (8.2)^{a,b,c}	58.4 (9.4)^{a,b,c}	46.7 (10.5)^{a,b}
4 h	0 cGy	71.3 (8.4)^{c}	73.1 (13.2)^{a,b,c}	62.5 (12.8)^{a,b,c}	70.5 (11.9)^{a,b}
	50 cGy	74.3 (13.5)^{c}	94.3 (11.1)^{b}	62.3 (12.2)^{a,b,c}	66.5 (13.4)^{a,b}
	200 cGy	76.3 (11.6)^{c}	95.1 (11.6)^{b}	65.1 (10.8)^{a,b,c}	55.9 (9.8)^{a,b}
6 h	0 cGy	64.4 (6.2)^{a,b,c}	84.5 (11.5)^{a,b,c}	63.0 (10.2)^{a,b,c}	76.5 (8.0)^{a,b}
	50 cGy	77.3 (10.6)^{c}	93.7 (12.3)^{b}	75.4 (15.5)^{b}	78.8 (16.8)^{a,b}
	200 cGy	88.0 (11.8)^{c}	105.7 (15.1)^{b}	92.7 (10.9)^{b,c}	62.2 (11.6)^{a,b}
8 h	0 cGy	74.6 (7.6)^{c}	102.6 (10.7)^{b}	65.9 (12.2)^{a,b,c}	87.4 (10.9)^{b}
	50 cGy	89.2 (15.0)^{b}	101.0 (16.7)^{b}	82.8 (19.5)^{b,c}	72.7 (15.8)^{a,b}
	200 cGy	91.4 (15.9)^{b}	108.1 (13.2)^{c}	93.7 (4.6)^{b,c}	67.7 (12.2)^{a,b}
24 h	0 cGy	47.0 (5.4)^{a,b,c}	68.3 (8.6)^{a,b,c}	52.6 (6.4)^{a,b,c}	51.2 (6.4)^{a,b}
	50 cGy	63.2 (8.3)^{a,b,c}	80.2 (13.2)^{a,b,c}	50.2 (13.1)^{a,b,c}	58.9 (12.7)^{a,b}
	200 cGy	72.2 (12.1)^{c}	80.1 (12.2)^{a,b,c}	61.4 (6.2)^{a,b,c}	53.6 (8.6)^{a,b}

Results are expressed as mean (s.e.m.); n = 10. "$^{+}$" indicates a Freidman's two-way ANOVA used for non-normal data. *P < 0.05, significant main effect of time point. Results within individual columns without a common superscript are different (P < 0.05).

Table 2.S2
Comparison of locomotor activity between restraint-10 and restraint-240 mice exposed to high-dose rate gamma radiation

Dose	Restraint time (min)	Time post irradiation				
		4 h	6 h	8 h	10 h	12 h
0 cGy	10	1654.8 (107.9)	1463.9 (83.7)	1240.5 (42.4)	865.6 (145.6)	605.4 (157.3)
	240	1559.1 (111.5)	1592.9 (101.8)	1606.7 (64.2)	1378.5 (77.9)*	753.6 (256.1)
50 cGy	10	1444.2 (89.1)	968.4 (147.6)	1040.4 (133.3)	660.6 (141.9)	544.8 (164.0)
	240	1457.3 (85.6)	1127.7 (82.0)	1342.4 (111.8)	1029.24 (149.0)	919.6 (156.3)
200 cGy	10	1401.6 (83.1)	950.5 (117.2)	882.7 (102.7)	658.4 (121.0)	530.3 (142.5)
	240	1955.9 (188.1)*	1743.9 (151.1)*	1404.2 (197.3)*	1119.9 (120.5)	977.2 (162.1)

*$P < 0.05$, restraint-10 versus restraint-240 within a given dose and time point.

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CHAPTER THREE

INDIVIDUALLY VENTILATED CAGES CAUSE CHRONIC LOW-GRADE HYPOXIA IMPACTING MICE HEMATOLOGICALLY AND BEHAVIORALLY

I ABSTRACT

Use of individually ventilated caging (IVC) systems for mouse-based laboratory investigation has dramatically increased. We found that without mice present, intra-cage oxygen concentration was comparable (21%) between IVC housing and ambient environment caging (AEC) that used wire top lids. However, when mice were housed 4-to-a-cage for 1 week, intra-cage oxygen dropped to 20.5% in IVC housing as compared to 21% for AEC housing. IVC intra-cage humidity was also elevated relative to AEC housing. Mice raised in IVC housing as compared to mice raised in AEC housing had higher RBC mass, hematocrit and hemoglobin concentrations. They also had elevated platelet counts but lower white blood cell counts. IVC mice relative to AEC mice had increased saccharin preference and increased fluid consumption but similar locomotion, food intake, social exploration and novel object recognition when tested in an AEC environment. Taken together, these data indicate that ventilated caging systems can have a 0.5% reduction from ambient oxygen concentration that is coupled to mouse red blood cell indices indicative of chronic exposure to a hypoxia. Importantly, IVC housing can impact behavioral testing for depressive-like behavior.

II INTRODUCTION

An important trend in laboratory rodent housing is the use of individually ventilated caging (IVC) systems. Purported advantages of IVC systems over conventional, ambient environment caging (AEC) (i.e. wire top, open air caging) are allergen and volatile organic compound (VOCs) reduction and the ability to increase animal population densities (1-3). In animal care/research personnel, allergy to laboratory animals can be as high as 44%, with a median time to allergy onset of less than 2 years (4-5). Allergen exposure can originate from sources such as urine, fur/pelt, saliva and serum proteins (6), and these allergens can contaminate the animal facility in both airborne particulate and fomite forms (7-8). VOCs such as ammonia have been identified as causative agents of "sick building syndrome", with animal care/research personnel reporting headache, nausea and fatigue (9). Educational training programs focused on personal hygiene and the use of personal protective equipment have reduced the incidence of laboratory animal-associated allergies, but with AEC the impact of such interventions has been modest (up to 22% of staff still developing allergies) (4). On the other hand, IVC housing has been shown to significantly reduce the important mouse-derived human allergen, murine urinary protein (7).

As we (10) and others (11) have reviewed, pre-experimental conditions are critical to rodent-based behavioral testing outcomes. The methodology by which mice are fed, handled and housed can dramatically impact a host of behaviors and like-behaviors including those that impact locomotion, food intake, learning/memory, social interaction, anxiety and depression (12). Little is known concerning how IVC housing affects mouse behaviors when compared to AEC housing. The single study published to date showed no effects of IVC housing in mice

during plus maze, open field, radial arm maze, acoustic startle or resident intruder tests (2). While others have investigated the impact of IVC housing on mouse behavior, these studies either used IVC system cages outside of the ventilation unit (13) or were comparing different IVC systems to one another (1). Importantly, no clear differences in IVC and AEC housing were seen or mechanism for behavioral change presented (1-2,13). In contrast, a cornucopia of data exists on the intra-cage microenvironmental differences between IVC and AEC housing with special attention paid to carbon dioxide, ammonia vapor and relative humidity (1,3,9,14-15). Surprisingly, intra-cage oxygen concentration in IVC housing has been ignored, although it is well known that in confined spaces with sealed ventilation systems like commercial airplanes (16), submarines (17) and space stations (18), oxygen concentrations can easily fall below 21%. In turn, hypoxia impacts a variety of physiologic functions and bioactives including behavior, as we have shown (19) and reviewed (20). In sum, no studies have reported on intra-cage oxygen concentration in IVC housing. Therefore, we examined intra-cage oxygen in IVC housing to determine its potential relevance to pre-experimental mouse physiology.

III METHODS

Materials. All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except as noted.

Animals and housing. Animal use was conducted in accordance with institutional guidelines for the care and use of laboratory mice. All experimental procedures were approved by the University of Illinois at Urbana-Champaign IACUC. All animals were housed in an AAALAC accredited laboratory facility as outlined in the Guide for the Care and Use of

Laboratory Animals (50). Male C57BL/6J mice, 3 wks of age, were obtained from The Jackson Laboratory (Bar Harbor, MN). Prior to shipping, mice had been housed in either IVC or AEC conditions by The Jackson Laboratory. Mice were group housed (4 per cage) in standard shoebox cages (28 cm x 17 cm x 12.5 cm) with wire top cage lids that were either open to the ambient environment (AEC) or attached to a positive-pressure Micro-VENT Mouse individually ventilated caging (IVC) system (Allentown, Inc., Allentown, NJ). All mice were allowed water and standard rodent chow (NIH-31 7013, Harlan Laboratories, Inc., Indianapolis IN) *ad libitum*. Regardless of housing method used, the room in which the mice resided was environmentally controlled on a 12:12 h dark:light cycle (2000 h-0800 h) at a temperature of 72º F (22 º C), relative humidity of 45-55% and 10-15 hourly air changes.

Oxygen, carbon dioxide, ammonia and humidity. Room and intra-cage air oxygen and carbon dioxide were measured using ProOx oxygen and ProCO_2 carbon dioxide sensors, respectively (Biospherix, Lacona NY). Humidity was measured using a digital hygrometer (Cat No. 11-661-18, Fisher Scientific, Pittsburgh PA). Ammonia was measured using a Kwik-Draw Sampling Pump (Cat. No. 488543, MSA, Pittsburgh PA) with 5-700 ppm Ammonia-specific sampling tubes (Cat. No. CH20501, Dräger Germany). Room and intra-cage air and humidity measurements were performed at 1400 h daily for 3 consecutive days. For intra-cage measurements, the sensors were located in the food hopper. All cages analyzed contained four mice. Measurements were randomized in regard to sensor placement within the room and to cage location within the cage racks.

Treatments and testing. Mice examined were between 8-12 wks of age and had spent 5-9 wks in AEC or IVC conditions. AEC mice were housed in AEC conditions (10-15 air changes/h)

and then treated and/or tested in AEC conditions. VCS mice were housed in VCS conditions (60 air changes/h) and then treated and/or tested in AEC conditions.

Hematology. After being housed in either AEC or IVC housing, mice were euthanized using carbon dioxide. Blood was drawn using post-mortem intracardiac puncture. A total approximate volume of 0.6-0.8 mL of blood was obtained from each mouse and placed into two separate (0.3-0.5 mL) pediatric EDTA anticoagulation microtainer tubes (Cat No. 365974, Becton Dickinson, Franklin Lakes NJ). Complete blood counts (CBC) and differentials were performed at Univesity of Illinois Veterinary Diagnotic Laboratory (Urbana IL) on a Abbott Diagnostics Cell Dyn 3700 automated hematology cell counter (Abbott Park IL).

Body mass and food and water consumption. Immediately prior to testing, mice were individually housed in AEC. Body mass and food and water consumption were measured daily at 1000 h by weight. Food and water consumption were determined from the weight of the water bottle plus water and the weight of the food container plus food before and after each 24 h data collection period by methods we have previously described (21). Briefly, the daily mass of the food or water in their respective containers were subtracted from the previous days mass, to determine amount consumed. Cage floors and bedding were carefully checked to account for food spillage and potential hoarding.

Movement. Movement was assessed by biotelemetry (Mini Mitter, Bend OR) as we have previously described (21). In brief, the surgical area (bench top) was cleaned with 70% ethanol. Mice were anesthetized via intraperitoneal injection of (80 mg/mL:12 mg/mL) ketamine:xylazine (Butler Schein Animal Health, Dublin OH) at 1.5 mL/kg body weight. Immediately preceding surgery, mice were injected intraperitoneally with buprenorphine at a dose of 0.05 mg/kg. Fur was shaved and the surgical site was aseptically cleaned and prepared using 10% povidone

iondine and 70% ethanol in three alternating wipes. Mice were kept on a heating pad during surgery and recovery. The abdomen was opened and a sterilized G2 e-mitter (Cat. No. 870-0010-01; Mini Mitter, Bend OR) was placed in the abdominal cavity along the saggital plane. 3-0 Vicryl violet braided dissolvable suture material (Cat. No. J393; Ethicon, Inc., Cornelia GA) was then passed through the silastic tubing attached to the outer wall of the e-mitter, and the e-mitter sutured to the body wall. The skin was then closed with 18/8 acid resistant, antimagnetic stainless steel 7.5 mm x 1.75 mm Michel suture clips (Cat. No. 12040-01; Fine Science Tools, Foster City CA) using forceps-style application. Immediately post-recovery, mice were individually housed in AEC and movement was recorded every hr for 5 days via under-cage ER-4000 receiver pads (Mini Mitter, Bend OR). Movement was quantified using Vital View data acquisition software (Mini Mitter, Bend OR).

Saccharin preference testing. Three days prior to saccharin preference testing (adaptation phase) mice were singly housed in AEC conditions adapted for two bottle water access. Both bottles contained water. After the adaptation phase, fluid bottles (randomized to right verses left) contained either water or a 0.4% sodium saccharin solution (Sigma-Aldrich, CN 4-7839) as we have previously described (22). Fluid consumption was recorded after 24 h. Water and saccharin consumption were recorded at 1000 h and determined by weight of the bottle and fluids before and after the 24 h data collection period.

Social exploration. 24 h prior to testing, AEC and VCS mice were individually housed in AEC and VCS conditions, respectively. After 24 h of new home cage acclimation, social exploration testing was conducted in AEC conditions, as we have previously described (19). At the time points of 0, 2, 4 and 6 h, a 3-4 week-old novel, conspecific juvenile mouse of the same sex (challenge mouse) was introduced into the home cage of the subject mouse. The challenge

mouse was placed in a 7.62 cm x 7.62 cm x 7.62 cm square metal mesh enclosure. Testing duration was 5 min and a new challenge mouse was used to test each subject mouse at every time point examined. Investigation/exploration was evaluated from the video record by a trained observer blinded to the pre-experimental housing conditions. Social exploration was considered as nose-to-enclosure contact. Video recording of animal behavior was performed under red light using a Sony HDR-XR500V Night Shot capable video camera (San Diego CA). Social exploration testing was initiated at 1000 h.

***Novel object recognition*.** One hour prior to testing, mice were individually removed from their home cage and placed for 5 minutes in a novel arena (home cage-sized with light bedding) containing two identical objects (large stainless steel bolts) positioned 10 cm apart at the short-side wall end 5 cm from the short side wall and 3.5 cm from the long-side wall. After training, mice were returned to their home cage for 1 hr. Testing was initiated by returning mice to the testing arena where one of the identical objects (familiar object) was replaced (randomized to right or left) by an unfamiliar object (novel object, a 5 mL microfuge tube). Investigative behavior was video recorded for 5 min and evaluated from the video record using EthoVision XT 7 (Noldus Information Technology, Leesburg VA). Percent investigation was calculated by dividing the time spent examining each object by the total time spent investigating both objects. Testing occurred 4.5 h after the beginning of the dark cycle (1200 h). Video recording of animal behavior was performed under red light using a Night Shot capable video camera.

Statistics. All data are presented as mean ± SEM. Data were analyzed using SAS 9.2 (SAS Institute, Inc., Cary NC). To test for statistical differences, a one-way or two-way ANOVA was used with or without repeated measurements where needed. Tukey's test was used for post-hoc pair-wise multiple comparison procedures. All statistical analysis included testing for time

point x housing type interactions, where appropriate. Statistical significance was denoted at $p<0.05$.

IV RESULTS

Air oxygen concentration is reduced in IVC housing when compared to AEC housing. Table 1 demonstrates that intra-cage air oxygen in IVC housing was 2.5% less than in AEC housing when mice were housed 4 per cage. Table 2 shows that intra-cage humidity was increased 44% in IVC housing compared to AEC housing when mice were housed 4 per cage. Carbon dioxide and ammonia concentrations were comparable between IVC and AEC housing (data not shown).

IVC mice show hematologic evidence of chronic exposure to low-grade hypoxia. Table 3 shows that IVC mice when compared to AEC mice had a 9.7%, 8.6% and 8.8% increase in RBC number, hemoglobin and hematocrit, respectively. Table 4 demonstrates that leukocytes and platelets were decreased 37.5% and increased 16.7%, respectively in IVC mice when compared to AEC mice.

IVC mice consume less water after transfer to an AEC environment. Table 5 shows that IVC mice during their first 24 h of individual housing in an AEC environment drink 15.1% less water compared to AEC mice transferred to individual housing in AEC conditions. Percent daily changes in body weight and food intake were not impacted by this housing switch. After 48 hr in an AEC environment, IVC mice water intake was comparable to AEC mice (Table 5).

IVC mice have an increased preference for saccharin but no change in locomotion, social exploration or novel object recognition. Fig.1A shows that IVC mice had a 10.9%

increase in saccharin preference compared to AEC mice (81.9 ± 3.6 *v.* 90.8 ± 1.2). This elevation was coupled to a 21.5% increase in total fluid consumption (water + saccharin solution) (9.1 ± 0.7 *v.* 11.1 ± 0.5) (Fig.1B). Table 6 demonstrates that, after implanting intraperitoneal biotelemetry probes, mouse movement for both AEC and IVC mice took 48 h to stabilize. Social exploration and novel object recognition was comparable between AEC and IVC mice (data not shown).

V DISCUSSION

To our knowledge, this is the first study to report a significant reduction in oxygen within IVC housing when compared to AEC housing (Table 1). In addition, the mean relative humidity was higher in IVC as compared to AEC housing consistent with previous reports (Table 2) (15). An expected physiologic response to reduced oxygen is increased red blood cell (RBC) mass and blood hemoglobin (23-24). Table 3 reflects such changes because IVC mice, compared to AEC mice, showed an increase in hematocrit as well as RBC and hemaglobin concentrations. Studies examining RBC physiology in mice at altitude have been performed with increases in hematocrit and RBC/hemoglobin concentrations seen (25). It is important to note that IVC housing is a low-grade hypoxia equivalent to an altitude of 90 m and normobaric (760 mm Hg) (26). At an altitude of 1760 m, the oxygen concentration is 84% that of sea level and the atmospheric condition is hypobaric (614 mm Hg) (26-27). In general, atmospheric pressure and inspired oxygen percentage decrease in a near linear fashion from 100% at sea level, with 50% of sea level oxygen available at 5500 m and 30% of sea level oxygen available at 8900 m (the peak of Mt Everest) (26). The likely mechanism for the increase in blood oxygen carrying capacity seen

in mice and men exposed to altitude is hypoxia-dependent up-regulation of erythropoietin which has been demonstrated in naturally occurring hypoxia (living at altitude (24)) and in experimentally-induced hypoxia (28).

Interestingly, total leukocytes were lower in IVC mice compared to AEC mice (Table 4). This drop in white cell count was not associated with a shift in leukocyte relative percentages. The reason for a lower white cell count in the face of a higher RBC mass is not clear. In humans, leukocyte numbers measured at sea level do not change significantly when re-measured after 8 months at an altitude of 3550 m (29). Unfortunately, data for leukocyte counts in mice housed at altitude is lacking. A possible cause of the lower WBC counts seen in IVC mice is the relative lack of ammonia in the IVC microenviorment (1). Von Borell et al. (30) has shown that pigs exposed to 35-50 ppm atmospheric ammonia for 19 days have increased absolute monocyte, lymphocyte and neutrophils counts. We, however, did not observe measurable ammonia in either IVC or AEC housing.

Platelet counts were higher in IVC mice relative to AEC mice. Previous work has demonstrated that humans exposed to 12.8% oxygen (normobaric) for 3 h had increased platelet counts that persisted for 24 h post-treatment (31). Mice exposed to 5.5-6.5% oxygen (normobaric) for 1-7 days had elevated platelet counts for the first 3 d of exposure before returning to normoxic control levels at day 4 and 5 of hypoxia exposure. Day 6 and 7 of hypoxia exposure showed significantly lowered platelet counts compared to normoxic control mice (32). Mechanisms of hypoxia-dependent thrombocytosis may potentially be gleaned from obesity research in that the obese state is associated with both elevated platelet counts in human females (33) and tissue hypoxia in animals (34). Bioactives implicated in thrombocytosis include leptin,

IL-6 and IL-1 which have all been shown to be increased in the obese state (35-36) and the hypoxic state (19,37-38).

We have previously shown that acute hypoxia leads to sickness behaviors in mice via a mechanism reliant on IL-1 (19,38). However, unlike mice exposed to 8% oxygen for 2 h, IVC mice had comparable locomotor activity, food intake, social exploration and novel object recognition as AEC mice. These findings are consistent with those of Mineu et al. (2), who showed that aggression, learning/memory and anxiety-like behaviors were unaffected by IVC housing. Water consumption and saccharin preference were significantly different between IVC and AEC mice (Table 5 and Fig.1). IVC mice had an enhanced preference for saccharin suggesting increased hedonism. This finding was unexpected due to human data showing an association between living at altitude and a higher rate of suicide (39-40). In humans, studies examining mood and cognitive function at altitude have been performed in locations between 3,000 m and 6,000 m above sea level (41-42). Acute mood disturbances and reduced performance of tasks have been reported in some individuals at altitudes as low as 3,000 m (42). Most persons will report impaired function at 5,000 m which has an oxygen concentration of 58% that of sea level (26,42). With ascent to altitude, a euphoric phase often occurs prior to the onset of mood depression (41-42). Additional evidence for the subjective feeling of euphoria can be found in reports dealing with "the choking game," which is a potentially life-threatening ritual used among adolescents around the world, which essentially consists of compression of the carotid arteries while holding one's breath (43). Since our mice were chronically exposed to low-grade hypoxia and then tested in a normoxic environment, the physiology modeled may be more akin to hyperoxygenation. Hyperoxygenation has been shown to increase memory and learning in rodent models of traumatic brain injury (44). However, the role of hyperoxygenation in

memory and learning remains clouded, as studies in rodent models of Alzheimer's Disease show it leads to cognitive impairment (45). In humans, fatigue is a reported complication of oxygen therapy, especially, hyperbaic oxygen therapy (46). The impact of increased oxygen on persons without hypercapnic respiratory problems are not known. Hyperventilation is associated with anxietal symptoms in humans (47) which are thought to be related to the impact of respiratory alkalosis on the CNS (48). Increasing the concentration of inhaled carbon dioxide by breathing into a paper bag is an effective way of decreasing anxiety associated with hyperventation while raising blood pCO_2 and, hence blood pH (49). Unfortunately, no work has been performed on depressive-like behaviors in mice associated with hyperoxygenation.

In sum, our findings indicate that IVC-housed mice could be used to model chronic low-grade hypoxia. Use of IVC-housing without a diligent examination of its impact on RBC mass and oxygen carrying capacity, is unadvised for those researchers investigating diseases tied to the hematopoietic system. How changes in oxygen effect mood is not understood but given the findings here warrants further study.

VI FIGURES

A) TABLE 3.1

Table 3.1

Oxygen (O_2) percentages in ambient room air and within AEC or IVC housing conditions.

	AEC	IVC
Ambient (room) O_2 (%):	**20.98 (0.03)[a]**	**20.97 (0.05)[a]**
Cage O_2 (%):	**20.98 (0.03)[a]**	**20.43 (0.05)[b]**
O_2 percentage change (%):	**0.00 (0.00)**	**-2.54 (0.10)***

Results are expressed as mean (s.e.m.), n=4-6/housing condition. *P < 0.0001, AEC O_2 percentage change (%) *v.* IVC O_2 percentage change (%). Results without a common superscript letter are significantly different (P < 0.0001).

B) TABLE 3.2

Table 3.2

Relative humidity (RH) percentages in ambient room air and within AEC or IVC housing conditions.

	AEC	IVC
Ambient (room) RH (%):	**26.67 (0.11)[a]**	**32.65 (0.75)[b]**
Cage RH (%):	**26.87 (0.09)[a]**	**47.28 (1.32)[c]**
RH percentage change (%):	**0.75 (0.37)**	**44.87 (4.18)***

Results are expressed as mean (s.e.m.), n=4-6/housing condition. *P < 0.0001, AEC RH percentage change (%) *v.* IVC RH percentage change (%). Results without a common superscript letter are significantly different (P < 0.0001).

C) TABLE 3.3

Table 3.3

Red blood cell (RBC) counts and related RBC parameters from AEC- and IVC-housed mouse blood draws.

	AEC	IVC
Red blood cells (M/µL):	**8.46 (0.09)[a]**	**9.28 (0.07)[b]**
Hemoglobin (g/dL):	**12.78 (0.13)[a]**	**13.88 (0.06)[b]**
Hematocrit (%):	**41.57 (0.39)[a]**	**45.23 (0.31)[b]**
Mean cell volume (fL):	**49.02 (0.15)**	**48.80 (0.31)**
Mean cell hemoglobin (pg):	**15.08 (0.11)**	**14.95 (0.10)**
Mean cell hemoglobin concentration (g/dL):	**30.75 (0.20)**	**30.68 (0.10)**
RBC distribution width (%):	**18.55 (0.56)**	**18.58 (0.26)**

Results are expressed as mean (s.e.m.), n=4-6/housing condition. Results within rows without a common superscript letter are significantly different ($P < 0.05$).

D) TABLE 3.4

Table 3.4

Leukocyte counts, differentials and platelet count/volume from AEC- and IVC-housed mouse whole blood draws.

	AEC	IVC
Leuckocytes (K/µL):	**2.99 (0.34)[a]**	**1.87 (0.17)[b]**
Neutrophils (%):	**7.14 (1.02)**	**10.63 (1.64)**
Lymphocytes (%):	**80.82 (6.12)**	**81.18 (1.99)**
Monocytes (%):	**8.43 (4.91)**	**5.17 (1.96)**
Eosinophils (%):	**0.96 (0.25)**	**0.97 (0.25)**
Basophils (%):	**2.64 (0.90)**	**2.07 (0.48)**
Platelets (K/µL):	**890.83 (37.30)[a]**	**1039.30 (27.35)[b]**
Mean platelet volume (fL):	**6.47 (0.14)**	**6.26 (0.09)**

Results are expressed as mean (s.e.m.), n=4-6/housing condition. Results within rows without a common superscript letter are significantly different ($P < 0.05$).

Table 3.5

Percent change in body weight, food and water consumption (g) following relocation from group-housed IVC or AEC cages to novel AEC housing 24, 48 and 72 h following relocation.

	24 h		48 h		72 h	
	AEC	**IVC**	**AEC**	**IVC**	**AEC**	**IVC**
Body weight	**0.68 (0.33)**	**0.51 (0.51)**	**-0.26 (0.55)**	**0.16 (0.32)**	**0.52 (0.28)**	**0.11 (0.68)**
Food intake	**3.92 (0.29)**	**3.90 (0.15)**	**3.80 (0.24)**	**3.98 (0.20)**	**3.53 (0.23)**	**3.53 (0.09)**
Water intake*#	**4.83 (0.15)^{a}**	**4.10 (0.12)^{b}**	**4.25 (0.17)^{a,b}**	**4.03 (0.18)^{b}**	**4.17 (0.18)^{b}**	**3.83 (0.19)^{b}**

Results are expressed as mean (s.e.m.), n = 6-12 per group. *$P < 0.05$, main effect of cage type; $^{\#}P < 0.05$, main effect of time point. Results without a common superscript letter are significantly different ($P < 0.05$).

F) FIGURE 3.1

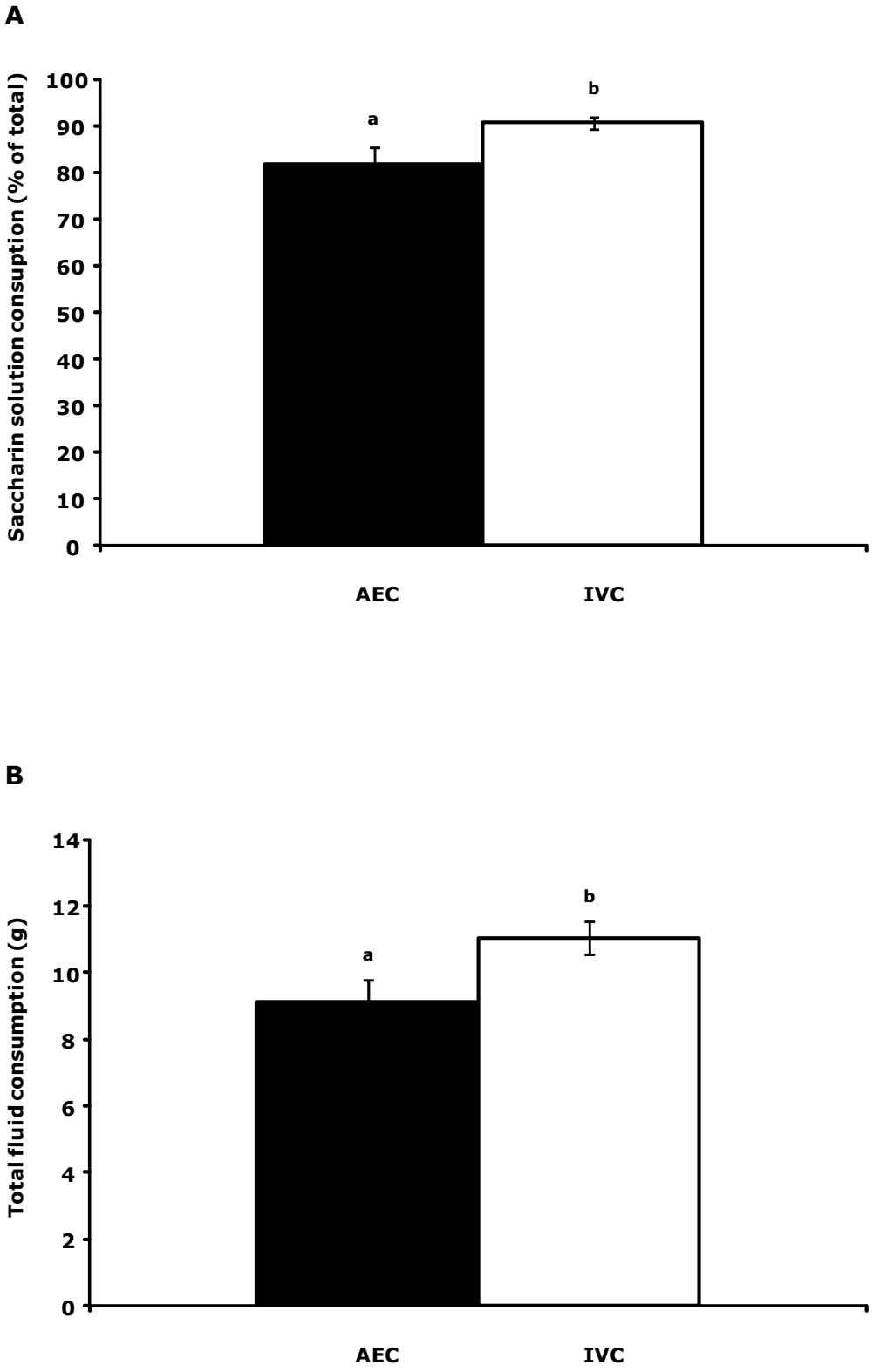
A
Saccharin solution consuption (% of total)
100
90
80
70
60
50
40
30
20
10
0
a
b
AEC
IVC
B
Total fluid consumption (g)
14
12
10
8
6
4
2
0
a
b
AEC
IVC

Figure 3.1. IVC mice show an increased preference for saccharin and consume more total fluid than AEC mice. (*A*) Results are expressed as percent saccharin consumed, means ± s.e.m., n = 12. Bars without a common superscript are significantly different ($P < 0.05$, AEC *v* IVC: 81.887 ± 3.600 *v*. 90.798 ± 1.235). (*B*) Results are expressed as total fluid consumed in grams, means ± SEM: n = 12. Bars without a common superscript are significantly different ($P < 0.05$, AEC *v*. IVC: 9.112 ± 0.678 *v*. 11.073 ± 0.488).

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